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13. ABSTRACT *(Maximum 200 Words)*

This proposal focuses on two key steps in the metastatic pathway: angioinvasion and tumor cell transmigration across the endothelium. HER2 amplification is associated with early tumor dissemination, rapid tumor progression and increased invasiveness. The overall objective of this proposal is to determine if HER2 signaling-induced production of Angiopoietin-2 (Ang-2) in breast cancer cells imparts a metastatic advantage,and to determine if overexpression of HER2 in human breast cancer is linked to angiopoietin-2 expression.

Specific aim #1 will determine if production of Ang-2 by the HER2 expressing cancer cells induces angioinvasion (by testing for microvessel dismantling) and transendothelial cell migration (by testing for endothelial cell retraction).

Specific aim #2 will determine if Angiopoietin-2 is co-expressed with HER2 in human breast cancers, and further to determine if Angiopoietin-2 expression is linked to a specific hetero-dimer of HER2 (EGFR, HER3, or HER4). We will employ Laser Capture Microdissection to precisely select cancer cells for study. After selection, RT-PCR technology will be used to detect expression of the mRNAs of interest.

The results of the experiments in this proposal will potentially lead to therapeutic interventions to block angiopoietin-2 from promoting tumor metastasis. These interventions may act synergistically with the Herceptin® antibody blockade of HER2. Additionally, this data may assist in selecting which patients would benefit from Herceptin ® antibody therapy.

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Introduction

HER2 overexpression is a poor prognostic indicator in breast cancer. HER2 amplification is associated with early tumor dissemination, rapid tumor progression, and increased invasiveness, implying that HER2 has a significant role in the metastatic phenotype. We have demonstrated that two key steps in the metastatic mechanism, angioinvasion and transendothelial migration, are augmented by HER2 expression, and we have linked Angiopoietin-2, a vascular destabilizing protein, to expression of HER2. The **objective** of this research is to determine if the metastatic advantage of HER2 expressing cancer cells is imparted by Angiopoietin-2 production, and further to determine if overexpression of HER2 is linked to Angiopoietin-2 expression. The **scope** of this research begins with two assays to test angioinvasion and endothelial cell retraction, a key step in transendothelial migration. Using several strategies, the research protocol tests tumor cell production of Angiopoietin-2 or blockade of Angiopoietin-2 to determine if Angiopoietin-2 modulates the metastatic steps in question. Further, breast cancer specimens are tested for concurrent expression of HER2 and Angiopoietin-2, and also correlated with stage and grade of the tumor. In addition, concurrent expression of related receptors (Epidermal Growth Factor receptor, HER3, and HER4) are also tested for correlation of Angiopoietin-2 expression.

Body

Specific aim #1 calls for testing the effect of manipulating Angiopoietin-2 production in MCF-7 breast cancer cells on in vitro models of key metastatic steps. The first series of experiments tested tumor cell induced endothelial cell retraction, a key step in tumor cell transendothelial migration. Angiopoietin-2 expression was manipulated in these cells using 3 techniques: 1) Stimulating or blocking HER2 signaling using Herceptin (a monoclonal antibody that binds to HER2 and prevents dimerization and signaling) or Heregulin β1 (a ligand for HER3 and HER4, which upon binding induces dimerization with HER2 and subsequent signaling;) 2) Direct application of Angiopoietin-2 into the assay or sequestration of tumor produced Angiopoietin-2 in the assay by treatment with soluble Tie-2/Fc receptor fusion protein; and 3) Angiopoietin-2 cDNA antisense transfection to decrease tumor cell production of Angiopoietin-2.

Experiment Series #1. Tumor cell induced endothelial cell retraction. These experiments tested whether Her2 signaling in MCF-7 breast cancer cells induced endothelial cell retraction during co-culture. Further, these experiments tested whether Ang-2 production by breast cancer cells was a key factor in the mechanism of EC retraction. MCF-7 cells or transfected MCF-7 cells that overexpress HER2 (HER cells) were used as the breast cancer model. Intact, 5-day-old monolayers of human iliac vein endothelial cells were used as the endothelial cell model. We used Heregulin β1 to stimulate Her2 signaling (induces Her2/Her3 dimerization and Her2 signaling) or Herceptin to block Her2 signaling. (Herceptin is a monoclonal antibody against Her2 that prevents dimerization and signaling.)

We have shown that HER cells induce a greater degree of endothelial cell retraction retraction, determined by A) number of tumor cells associated with endothelial cell retraction events, and B) the percent of subendothelial matrix that is exposed by retracting endothelial cells. (1)We have also shown that HER2 signaling induces endothelial cell retraction. (1) MCF-7 or

HER cells were pretreated with Herceptin or Heregulin β1 to block or stimulate HER2 signaling. The MCF-7 cells were then cocultured onto the intact human endothelial cells. Heregulin β1 treatment greatly increased the degree of endothelial retraction induced by coculture with MCF-7 cells. Conversely, endothelial cell retraction was effectively arrested by tumor cell treatment with Herceptin to block HER2 signaling. (1)

We have shown that HER cells produce more Angiopoietin-2 than MCF-7 parental cells (2). These results implicated Ang-2, a vascular destabilizing protein involved in angiogenesis, in the mechanism of breast cancer cell-induced endothelial cell retraction. To test this hypothesis, Angiopoietin-2 was directly applied in escalating doses to the intact endothelial monolayers in the retraction assay. Further, MCF-7 cells were pretreated with soluble Tie2/Fc receptor fusion protein (sTie2/Fc) to bind and sequester tumor cell released Angiopoietin-2, to prevent Angiopoietin-2 interaction with the Tie-2 receptors on the endothelium. Low doses of Angiopoietin-2 failed to induce significant retraction, but at 200 ng/ml, Angiopoietin-2 induced a great degree of retraction, on scale with HER cells ($p < 0.01$ vs MCF-7). (3)(Low dose failure may be attributable to failure to overcome intrinsic Angiopoietin-1 stimulation of the Tie-2 receptor, to which it binds with equal affinity. The role of Angiopoietin-1 is endothelial stabilization, an opposite effect of Angiopoietin-2.) Increasing doses of sTie2/Fc to sequester Angiopoietin-2 significantly altered the ability of MCF-7 cells to induce endothelial cell retraction at a dose of 200 ng/ml sTie2/Fc ($p < 0.05$ vs MCF-7). (3)These experiments suggest that Angiopoietin-2 is likely a factor in tumor cell induced endothelial cell retraction. To complete these experiments, we will test MCF-7 cells depleted of Ang-2 expression by antisense Ang-2 transfection for the ability to induce endothelial cell retraction. If Ang-2 depleted cells fail to induce EC retraction, the findings will strongly suggest a key role of Ang-2 in this mechanism. We have 3 clones with stable transfection. To test for Ang-2 depletion, however, we had to generate an appropriate anti-Ang-2 monoclonal antibody for the application using SELDI (Surface Enhanced Laser Desorption/Ionization) technology. Because the amount of Ang-2 protein produced by these cells is low, definitive depletion could not be accurately determined by Western blot, and will be screened by SELDI. We now have a functional anti-Ang-2 antibody, and the SELDI experiments will be performed soon. Additionally, we now have available a specific RNA aptamer that exclusively binds Ang-2. We will be able to test for specific sequestration of Ang-2 without concurrent sequestration of Ang-1 (as was seen with sTie2/Fc). This will more specifically detail the role of Ang-2 in this mechanism of EC retraction. (These data will be included in a revision of the manuscript accepted for publication by the Annals of Surgical Oncology.)

Experiment series #2. The next series of experiments evaluated Her2 signaling and the role of tumor produced Angiopoietin-2 in the mechanism of angioinvasion through microvessel dismantling. Angioinvasion was studied using a 3 dimensional in vitro microvessel-dismantling assay of isolated rat microvessels embedded in collagen I gel. (1)We have shown that microvessels dismantle upon exposure to MCF-7 cells or HER cells. After coculture with these cells, embedded microvessels demonstrate areas of discontinuity, with architectural dismantling. We compared MCF-7 cells with HER cells, which express significantly more Angiopoietin-2 (4). HER cells induce a significantly more rapid and more extensive effect ($p < 0.05$ vs MCF-7). To further implicate HER2 signaling as a mechanism for this metastatic step, we pretreated

MCF-7 cells with Herceptin or Heregulin β1 to block or induce HER2 signaling. Blockade or stimulation of HER2 signaling can dose dependently limit or enhance tumor cell induced microvessel dismantling ($p < 0.01$). We further demonstrated that other HER2 expressing breast cancer cell lines can induce microvessel dismantling, and in at least one additional line the effect is blocked by Herceptin blockade of HER2 signaling. (4) We also tested the direct application of Angiopoietin-2 protein to induce microvessel dismantling. Further, we used sTie2/Fc to sequester tumor produced Angiopoietin-2. Microvessels in the dismantling assay were exposed to Angiopoietin-2 protein in increasing doses up to 200 ng/ml. No significant induction of microvessel dismantling was identified. We also treated the MCF-7 cells with sTie2/Fc to sequester Angiopoietin-2, and exposed the microvessels to these pretreated cells. sTie2/Fc dose-dependently-inhibited induced dismantling, reaching significance at 200 ng/ml ($p < 0.01$) although this effect was not dramatic. It appears that although Ang-2 may be key to EC retraction, the mechanism of Her2-induced microvessel dismantling involves other Her2-signaling upregulated factors.

Experiment series #3. These experiments were not described in the Statement of Work, but were designed to further elucidate the mechanism of HER2 signaling and Angiopoietin-2 induction of endothelial cell retraction. We postulated that endothelial cells retract after the binding of Angiopoietin-2 to the Tie2 receptor on the endothelial cell induces the dissociation of the catenin proteins from vascular endothelial (VE) cadherin. These proteins are key structural proteins of the adherens junctions of the endothelium. Under appropriate stimulation, a sequential dissociation of γ, β, and then α catenin from VE cadherin breaks the adherens junction link to the cytoskeleton, resulting in retraction and rounding of the endothelial cell. In these experiments, we tested intact human endothelial cell monolayers for dissociation of the catenins from VE cadherin after exposure to MCF-7, and further tested the MCF-7 cells after treatment with Herceptin and Heregulin β1 to manipulate HER2 signaling in these cells. Using immunoprecipitation of VE cadherin after exposure of the monolayer to tumor cells, we determined the quantity of the catenins, which remained associated with VE cadherin by Western blot analysis. Figure 1 shows the quantity of the catenins linked to VE cadherin over time of exposure to MCF-7 cells. Western blots were digitized and the densitometric intensity was determined and compared to control. The data is reported as percent of control (untreated) monolayers. A time dependent loss of associated catenins is clearly demonstrated, with greater than 90% loss of γ catenin seen at 24 hrs ($p < 0.01$). Further, HER2 signaling regulation using Herceptin and Heregulin β1 significantly altered the dissociation curve (Figure 2). A 50% reduction in γ catenin dissociation was seen at 24 hrs after treatment with Herceptin. Heregulin β1 significantly augmented MCF-7 induced γ catenin dissociation ($p < 0.05$), achieving equivalence with the result of HER cell induction of catenin dissociation. (Recall that HER2 signaling modulations alters the tumor cell production of Angiopoietin-2). These results imply that the mechanism of HER2 signaling induced endothelial cell retraction likely includes dissociation of the adherens junction proteins, with loss of continuity with the cytoskeleton. Additional experiments tested Angiopoietin-2 sequestration with sTie2/FC from MCF-7 cells in coculture with endothelial monolayers. After pretreatment with increasing doses of Tie2/Fc, MCF-7 cells were cocultured with endothelial monolayers. After immunoprecipitated with anti-VE cadherin antibody, Western blot analysis of the immunoprecipitate for γ catenin was performed. Figure 3 shows that sequestration of Angiopoietin-2 with sTie2/Fc significantly reduced the γ catenin dissociation induced by MCF-7 cells back to 50% of control ($p < 0.05$ vs

MCF-7), similar to Herceptin treatment. This experiment further implicates tumor cell produced Angiopoietin-2 as part of the mechanism of the HER2 signaling induced, metastatic phenotype. We are still testing the direct application of Angiopoietin-2 protein to induce γ catenin dissociation. Further, we will also test the aptamer that specifically binds Ang-2 to support this proposed mechanism.

Specific Aim #2 calls for the determination of concurrent expression of HER2 and Angiopoietin-2 in breast cancer specimens with the aim to determine if HER2 expression is linked to Angiopoietin-2 expression in breast cancer. The method uses laser capture microdissection as described in the proposal protocols. Because HER2 is the signaling subunit of heterodimers with other types I growth factor receptors (EGFR, HER3, and HER4) we will also determine relative levels of these receptors in the breast specimens. To date we have collected over 50 cancer specimens, and have tested 14 cancers. We have also tested three normal breast specimens along with placenta as a positive control. Figure 4 illustrates one experiment, demonstrating co-expression of Her2 and Ang-2. Of 14 breast cancers, 7 over-express HER2. All 7 cancers that over-express Her2 also express Angiopoietin-2. No correlation has been statistically quantitated to date to link co-expression of other HER receptors to Ang-2 expression, but Her3 expression appears to be expressed in Ang-2 producing cancers (see later discussion). These data support the HER2 link to Angiopoietin-2 production.

Recent publications have indicated that Her2 signaling (stimulated by Heregulin β1) upregulate expression of VEGF (5, 6). Because Ang-2 is also a key factor in angiogenesis, particularly in the presence of VEGF, we expanded the scope of the research to include additional angiogenic factors. Figure 5 shows gene expression in several breast cancer cell lines, with relative expression of several key angiogenic factors. MCF-7 (2 separate strains, one estrogen dependent "MCF-7b", and one independent "MCF-7") MDA-MB-175, and SkBR3 cells all express Ang-2, while MDA-MB-231 cells do not. The –231 cells also do not express significant quantities of VEGF. These cells also do not express Her3 or Her4, which are key heterodimers for Her2 signaling. We had prior published that –231 cells did not express Ang-2 although they expressed Her2 (2). It appears plausible that these cells do not express Ang-2 because of lack of expression of Her3 or Her4. (Additionally, published reports indicate Her2/Her3 dimers may be responsible for VEGF upregulation in breast cancer cells.) Also of interest, the –175 cells express Her2 and Her3, but not significant quantities of Her4, yet express VEGF and Ang-2. Further, this data indicates that the MCF-7 cells have limited expession of Ang-1, a vascular stabilizing protein that competitively binds with Ang-2 to the Tie2 receptor. We postulate that the lack of Ang-1 expression in MCF-7 cells contributes to the phenotypic expression of induced EC retraction seen in our model. Additional experiments are planned with design to identify the in vitro angiogenic induction capability of these cells lines, and to test these additional hypotheses.

Because the data implicates an angiogenic response in these cells from Her2 signaling, we further expanded the scope of this project to perform microarray analysis for angiogenic factors in these cell lines (Figs. 6,7). We tested the MCF-7 cells for angiogenic factor gene expression using a GEArray chip containing 96 angiogenic genes. The microarrays were also run on RNA extracted from MCF-7 cells after treatment with Heregulin β1 to stimulate Her2 signaling, and Herceptin to block Her2 signaling. Additionally, stimulation with Heparin Binding-Epidermal Growth Factor (HB-EGF, which induces EGFR dimerization and signaling and Her4 dimerization to EGFR with possible alternate signaling) was also performed. Although

this data is currently being evaluated, preliminary results indicate significant upregulation of several angiogenic factors after Heregulin β1 stimulation (including FGF2, neuropilin 1, and TIMP1). HB-EGF stimulation also upregulated angiogenic factors including FGF1, FGF2, neuropilin 1, and VEGFB. Herceptin treatment demonstrated down regulation of multiple factors including Ang-2, PDGF-B, MMP2 and MMP9, IL-8, and integrin β3. Microarray analysis for angiogenic factors is planned for several human cancers to identify correlation with the cell lines. Although this expanded scope can not be completed in the time duration of this Her2 signaling project, the preliminary data may support justification for additional study of the mechanism of the Her2-signaling induced metastatic phenotype.

Key Research Accomplishments

- Determined that HER2 signaling induces a metastatic phenotype in breast cancer involving endothelial cell retraction (as a key step in transendothelial migration) and microvessel dismantling (as a potential avenue of angioinvasion.)
- Determined that Angiopoietin-2 can induce endothelial cell retraction, and is likely a key factor in the mechanism of the HER2 signaling induced metastatic phenotype
- Determined that the mechanism of endothelial cell retraction involves dissociation of the catenin proteins from VE cadherin, and loss of adherens junctional linkage to the cytoskeleton
- Determined that Angiopoietin-2 is likely involved in the mechanism of HER2 signaling induced microvessel dismantling, but is not the only factor in this mechansim.
- Identified that concurrent expression of HER2 and Angiopoietin-2 exists in breast cancer, although alternate-signaling pathways may also influence Angiopoietin-2 production in cancer cells.
- Identified that concurrent expression of Her3 is likely required for Angiopoietin-2 (and VEGF) expression in breast cancer cells.
- Identified that several angiogenic factors appear to be regulated by Her2 signaling in MCF-7 cancer cells.

Reportable Outcomes

1. Carter WB. HER2 signaling-induced microvessel dismantling. *Surgery* 2001;130:382-387.
2. Carter WB, Ward MD. HER2 signaling-induced microvessel dismantling. Abstract. Presented at the Society of University Surgeons, February 10, 2001, Chicago, IL.
3. Carter WB, Hoying JB, and Williams SK. HER2 overexpression enhances tumor cell transendothelial migration. Abstract. Presented at the Society of Surgical Oncology, March 12, 2001, Washington, D.C.
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5. Carter WB, Ward MD, Hoying JB. Mechanism of HER-2 induced endothelial cell retraction. *Annals of Surgical Oncology.* Accepted for publication.
6. Carter WB, Small G, Ward MD. Mechanism of Her2-induced endothelial cell retraction. Poster abstract. Presented at the Era of Hope, September 25-28, 2002, Orlando, FL.

Conclusions

The work to date has substantially increased the knowledge available about the mechanisms involved in the development of a metastatic phenotype associated with HER2 overexpression. We have shown that at least two metastatic mechanistic pathways are enhanced by HER2 signaling; 1) endothelial cell retraction and transendothelial migration, and 2) microvessel dismantling as a portal for angioinvasion. Further, these metastatic pathways appear to involve Angiopoietin-2, a vascular destabilizing protein. The work presented identifies that the Angiopoietin-2/Tie-2 receptor pathway is likely a key intermediary step in the metastatic phenotype, and a worthy therapeutic target. Further, the determination of Angiopoietin-2 expression in breast cancer may suggest an appropriate tumor marker indicating greater metastatic or angiogenic potential. The remaining experiments designed in this study are likely to offer additional insights into these mechanisms, and perhaps elucidate other potential clinical targets or markers.

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FIGURE LEGENDS

1. Anti-VE-cadherin immunoprecipitation of human iliac vein endothelial (HIVE) monolayers exposed to MCF-7 cells with Western blot analysis of VE-cadherin associated catenins over time. Western blots were digitized and densitometric intensity determined. The data is reported as % of control (untreated) HIVE monolayers. Loss of immunoprecipitated γ catenin is significant by 6 hr ($p < 0.05$) and nearly complete by 24 hr. Loss of immunoprecipitated α and β catenin are significant by 24 hr ($p < 0.001$).
2. Anti-VE-cadherin immunoprecipitation of HIVE monolayers exposed to MCF-7 or HER cells, or MCF-7 cells pretreated for 24 hrs with Heregulin β1 (100 ng/ml) or Herceptin (100 ng/ml) to induce or block Her2 signaling. The data is reported as % of control (untreated) HIVE monolayers. Heregulin β1 induced a greater loss of immunoprecipitated γ catenin compared to untreated MCF-7 cells at all time points ($p < 0.05$). Herceptin profoundly limited the dissociation of γ catenin ($p < 0.05$ at 6 and 12 hrs vs untreated MCF-7) with 50% reduction at 24 hrs ($p < 0.001$). HER cells also induced dissociation of γ catenin to a similar degree as Heregulin β1 treatment ($p < 0.05$ vs untreated MCF-7). * Data not obtained.
3. Anti-VE-cadherin immunoprecipitation of HIVE monolayers exposed to MCF-7 cells pretreated for 24 hrs with sTie2/Fc to sequester tumor cell produced Angiopoietin-2. Significant reduction of dissociated γ catenin was seen at 500 ng/ml compared to untreated MCF-7 ($p < 0.05$).
4. RT-PCR of a breast cancer specimen. Panel A demonstrates RT-PCR using specific primers for EGFR, Her2, Her3, and Her4, as well as Ang-2, VEGF and actin (for quantification.) Laser Capture Microdissection was performed on the tumor to avoid vascular cell contamination (panel B). This tumor represents 14 tumors studied to date. Note coexpression of EGFR, Her2 and Her3, with limited expression of Her4. This tumor strongly expresses Ang-2, with expression of VEGF.
5. Gene expression of the Her family of receptors and co-expression of angiogenic factors in selected breast cancer cell lines using specific primers and RT-PCR. MCF-7 (both estrogen dependent (B) and estrogen independent), SKBR3, and MDA-MB-175 cells express Her2, EGFR, and Her3, and co-express VEGF and Ang-2. MDA-MB-231 cells do not express Her3, and do not co-express VEGF nor Ang-2. Placental growth factor (PLGF) is expressed only by MCF-7 (estrogen independent) and SKBR3 cells (which express all 4 Her receptors). MCF-7B (estrogen dependent) cells did not express PLGF. Platelet derived growth factor B (PDGF-B) was universally expressed in these cell lines. Angiopoietin-1 (ANG-1), a factor involved in vascular stability and maturation of neo-vessels, was expressed by all the estrogen independent cell lines (but not MCF-7B).
6. Gene micro-array using GEArray angiogenesis chips containing 96 angiogenesis associated genes. MCF-7 cells alone or treated for 24 hrs with Heregulin β1 (HRG, 100 ng/ml, which stimulates Her2-Her3 dimers and signaling) or Heparin Binding-Epidermal Growth Factor (HB-EGF, 100 ng/ml, which stimulates EGFR-EGFR or EGFR-Her4 dimers and signaling) were tested for angiogenesis associated genes. Several genes were upregulated, including significant upregulation of several angiogenic factors after Heregulin β1 stimulation (including FGF2, neuropilin 1, and TIMP1). HB-EGF stimulation also upregulated angiogenic factors including FGF1, FGF2, neuropilin 1, and VEGFB.

7. Gene micro-array using GEArray angiogenesis chips containing 96 angiogenesis associated genes. MCF-7 cells alone or treated for 24 hrs with Herceptin (100 ng/ml, to block Her2 signaling). Herceptin treatment demonstrated down regulation of multiple factors including Ang-2, PDGF-B, MMP2 and MMP9, IL-8, and integrin β3.

HER2 regulatory control of angiopoietin-2 in breast cancer

W. Bradford Carter, MD, *and* **Michael D. Ward, BS,** *Norfolk, Va*

Background. *HER2 overexpression is a marker of aggressive breast cancer. Tumors that overexpress HER2 induce endothelial cell retraction and endothelial destabilization. Because angiopoietin-2 (Ang-2) also destabilizes microvessels, we postulated that HER2 signaling upregulates Ang-2 as a mechanism of angioinvasion.*

Methods. *We tested human breast cancers and breast cancer cell lines for coexpression of HER2 and Ang-2 with Northern blot, reverse transcriptase–polymerase chain reaction, and enzyme-linked immunosorbent assay. Further, we manipulated HER2 signaling with 100 ng/mL MAbHu HER2 (Herceptin; Genentech, San Francisco, Calif) and Heregulin β1 (100 ng/mL; R&D Systems, Inc, Minneapolis, Minn) to test for HER2 regulation of Ang-2 production.*

Results. *Three of 4 breast cancer cell lines expressed HER2 protein and Ang-2 mRNA. HER cells, a stably transfected cell line that overexpresses HER2 6-fold, showed a 430% increase in Ang-2 mRNA compared to parental MCF-7 cells. Heregulin β1 stimulation of HER2 signaling in MCF-7 cells increased Ang-2 by 20% ($P < .05$). HER2 signaling blockade with 100 ng/mL Herceptin reduced Ang-2 mRNA 90% ($P < .001$). Five of 11 cancers expressed both HER2 and Ang-2; 2 cancers expressed only Ang-2.*

Conclusions. *We conclude that human breast cancers express Ang-2. HER2 signaling appears to regulate Ang-2 expression, although other signaling pathways may also regulate Ang-2. Ang-2 may be a therapeutic target in these cancers and may define which patients would benefit from Herceptin therapy. (Surgery 2000;128:153-8.)*

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OVEREXPRESSION OF HER2 (neu, C-erb B2), a type I growth factor receptor, imparts an aggressive metastatic phenotype and poor prognosis in breast cancer. In fact, HER2 is the most powerful predictor of disease-free and overall survival after lymph node status.[1-3] HER2 expression in breast cancer cells is inversely related to the estrogen receptor.[4] It is believed that as the cell loses estrogen receptor expression, the cellular functions are replaced by upregulation of the type I growth factor receptors, which are involved in growth, differentiation, and survival.[5,6]

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This family of tyrosine kinase receptors is expressed in a wide range of different cell types. Four distinct members have been identified: EGFR (C-erb B1), HER2 (C-erb B2, neu), HER3 (C-erbB3), and HER4 (C-erbB4). The ligands for these receptors are the neuregulins, which bind to HER3 or HER4, causing heterodimerization with HER2.[7,8] HER2 is the signaling subunit and has no independent ligand. Antibody blockade of HER2, preventing heterodimerization, eliminates neuregulin-stimulated signaling.[9-12]

HER2 expression imparts a metastatic advantage to the cell. Blockade of HER2 dimerization and signaling delays return to a tumor growth phase and induces tumor regression in breast cancers that express HER2.[13,14] To date, very little information has been published that establishes the metastatic mechanisms that are enhanced by the cellular expression of this receptor. We have postulated that the expression and signaling of HER2 is linked to cellular production of a factor that influences the metastatic phenotype. Recently, Oh et al[15] have identified downstream alterations in gene expression as a direct consequence

of HER2 signaling. In previous studies, we have identified that HER2 expression and signaling significantly augment cell transmigration across intact endothelium and that the mechanism appears to involve a secreted product that induces endothelial cell (EC) retraction by loss of EC-EC contact (unpublished data). Because of the vascular activity of the secreted product, we have postulated that angiopoietin-2 (Ang-2) may be a factor involved in the HER2-enhanced metastatic phenotype.

Ang-2 is a recently discovered protein that is involved in the process of angiogenesis. This process is poorly understood but is known to involve EC dissociation, shape change, and invasion of the supporting matrix.[16] In adult tissues, angiogenesis is a rare event, except in reproductive tissues (ovary, uterus, and placenta). When angiogenesis is initiated, the ECs dissociate, the microvessels destabilize, and new blood vessels are formed from existing blood vessels. Recently, a family of EC-specific tyrosine kinase receptors has been identified, Tie-1 and Tie-2. The ligands for Tie-2, angiopoietin-1 (Ang-1), and Ang–2, are antagonistic although they bind with equal affinity.[17] Ang-1 appears to function in the stabilization of vessels and is widely expressed in tissues.[18] Conversely, Ang-2 is only expressed in tissues under menstrual control, which are actively angiogenic.[17] Ang-2 binding blocks the function of Ang-1. The result is destabilization of the vessel with dissociation of EC-EC junctions. In the ovary, Ang-2 is expressed at sites of vessel regression and invasion,[17] where microvessels dismantle. In transgenic mice that overexpress Ang-2, blood vessels were discontinuous with islands of ECs. ECs were detached from the basement membrane and were not typically elongated but rounded.[17] These observations appeared identical to EC responses to coculture with HER2-expressing cancer cells, which cause EC-EC dissociation and EC retraction (unpublished data).

We hypothesize that HER2 signaling induces the cellular production of Ang-2. Ang-2 binding to the Tie-2 receptor induces EC retraction and creates a portal for transendothelial migration of tumor cells into secondary tumor sites. In this study, we tested MCF-7 breast cancer cells (low expressors of HER2 with a low metastatic potential) for the production of Ang-2. We also manipulated HER2 signaling in these cells, using MAbHu Her2 (Herceptin; Genentech, San Francisco, Calif) to block and heregulin-β1 to stimulate HER2 dimerization, and evaluated the downstream cellular response of production of Ang-2 messenger RNA. We further tested other breast cancer cell lines and human breast cancer specimens for the coexpression of HER2 and Ang-2 mRNA.

MATERIAL AND METHODS

Cell lines. For these experiments, we used MCF-7, SK-BR3, MD-175, and MD-231 breast cancer cell lines (American Type Culture Collection, Rockville, Md). These cell lines are maintained in high glucose Dulbecco's modified eagle medium with 10% fetal bovine serum, with penicillin (100 U/mL) and streptomycin (100 μg/mL), pH 7.4 at 37°C, 5% carbon dioxide. In addition, we used HER cells, an established cell line derived from MCF-7 cells (C. Benz, San Francisco, Calif), that overexpresses HER2 by stable transfection with HER2.[19] These cells are also maintained in the Dulbecco's modified eagle medium with 10% fetal bovine serum, with the addition of G418 (300 μg/mL) selection medium.

RNA preparation. Total RNA was purified from either cell lines or tissues with the use of the RNEasy Mini kit (Qiagen, Inc, Valencia, Calif) according to the manufacturer's protocol. Typically, 1×10^7 cells were harvested from subconfluent cultures or 20 mg of frozen powdered tissues and used as starting material. Typical yields were 60 to 100 μg total RNA.

Northern blot analysis. Total RNA (10 μg) was electrophoresed on 1.2% formaldehyde #3-[n-morpholino] propane sulfonic acid–agarose gels and transferred to nitrocellulose by capillary elution in 10 × standard saline citrate buffer (SSC) according to established protocols. RNA was fixed to the membrane by UV crosslinking. Membranes were hybridized overnight at 50°C at a probe concentration of 30 ng/mL. Blots were washed twice for 10 minutes in 2 × SSC/0.1% sodium dodecylsulfate (SDS; room temperature), followed by a 10 minute 0.5 × SSC/0.1% SDS wash at 65°C. Final washes were in 0.1 × SSC/0.1% SDS for 15 minutes at 65°C. Ang-2 probes were randomly primed; digonigenin-dUTP–labeled polymerase chain reaction (PCR)–generated fragments of 510 bp were derived from human Ang-2 complementary DNA (provided by Dr George Yancopoulos, Regeneron Pharmaceuticals, Tarrytown, NY). Blots were developed with a DIG Nucleic Acid Detection kit (Roche Molecular Biochemicals, E Rutherford, NJ) and exposed to x-ray film (Fuji RX; Fugi Medical Systems, Inc, Stanford, Conn).

Reverse transcriptase–polymerase chain reaction analysis. For each cell line or tissue specimen, 2.0 μg of RNA was reverse-transcribed (RT) and PCR amplified with the Access RT-PCR system (Promega Corp, Madison, Wis) according to manufacturer's protocol. Each reaction was first heated to 45°C for 45 minutes (RT) followed by a 2-minute RT denaturation step at 95°C. Subsequent cycles

were as follows: 1 minute at 95°C, 1 minute at 56°C, 2 minutes at 68°C, and 1 minute at 56°C. Final elongation was at 68°C for 7 minutes, followed by a 4°C hold. For semiquantitative PCR analysis, 10 μL aliquots from each reaction were removed at 3-cycle intervals, starting at cycle 22 and ending at cycle 35. Linearity was usually observed between 25 and 31 cycles. The Ang-2 primers used were [5′-CCGCTCGAATACGATGACTC-3′] (forward) and [5′-CGTTTGCTCAGCTGTTTGGT-3′] (reverse). To normalize Ang-2 mRNA in these samples, parallel reactions for β-actin mRNA with the use of commercially available primers (Promega) were performed under identical cycle conditions. With image analysis software (SigmaScan Pro; Jandel Scientific, San Rafael, Calif), relative abundance of Ang-2 was calculated from digital images of the gels by the assignment of the highest individual β-actin mRNA signal density as 100%. The numeric increase or decrease in relative β-actin signal from sample to sample was applied to the Ang-2 signal to quantitate the Ang-2 mRNA. Statistical analysis used the Student *t* test between groups.

Determination of HER2 and Ang-2 in tissue. Human breast cancer specimens (gift of G. Wright, Virginia Prostate Center, Norfolk, Va) were flash-frozen in liquid nitrogen, ground with a mortar and pestle, and resuspended in the provided enzyme-linked immunosorbent assay (ELISA; Ontogeny Research Products, Cambridge, Mass) diluent to approximately 10 mg/mL. The protein concentration was determined by standard Bradford protein assay, and the specimens were further diluted to 100 μg/mL. In addition to tumor specimens, 3 normal human breast specimens were obtained from breast reduction surgery, snap-frozen, and processed as described earlier. HER2 protein levels were determined by ELISA following the manufacturer's instructions. Total RNA was extracted from the powdered cancer and breast specimens and subjected to RT-PCR for Ang-2 as described earlier. Human tissue was used in this study with appropriate Institutional Review Board approval.

HER2 signal regulation assay. MCF-7 cells were subjected to 24-hour exposure with 1, 10, or 100 ng/mL Herceptin or 1, 10, or 100 ng/mL Heregulin β1 (100 ng/mL; R&D Systems, Inc, Minneapolis, Minn) or vehicle in the tissue culture medium described. Total RNA was extracted with the RNEasy minikit (Qiagen, Inc, Valencia, Calif) and Ang-2 mRNA determined by semiquantitative RT-PCR or Northern blot analysis. RNA from human placenta was used as a positive control for Ang-2, and normal human breast was used as a negative control. Statistical analysis used the Student *t* test between groups.

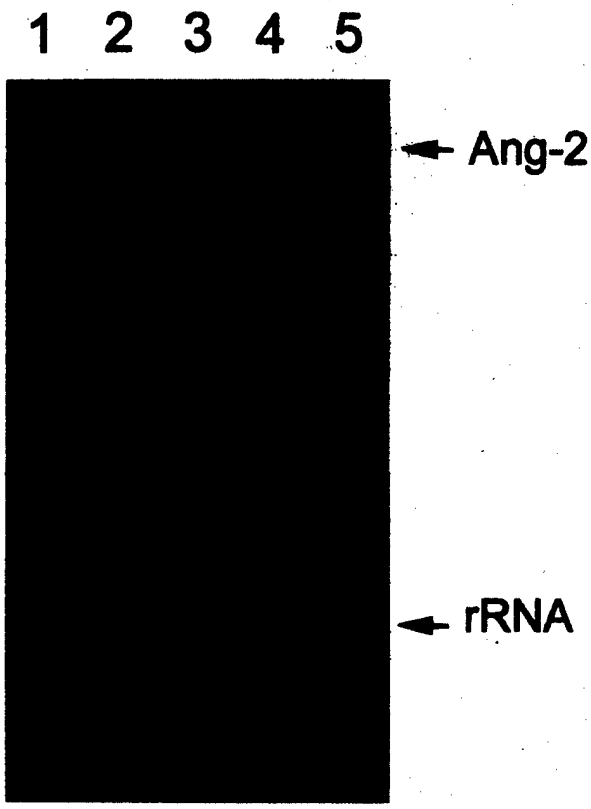

Fig 1. Northern blot analysis of Ang-2 expression in MCF-7 cells. Total RNA (10 μg) from MCF-7 cells either untreated (**lane 3**), treated for 24 hours with either Herceptin (**lane 4**) or heregulin (**lane 5**) was electrophoresed on a 2% formaldehyde-agarose gel, and the blot was probed with a 550-bp Ang-2 probe. Human placenta total RNA was used as a positive control (**lane 2**). Normal human breast epithelium was used as a negative control (**lane 1**). Levels of 18S RNA (*rRNA*) were used as a control for RNA integrity and loading consistency.

RESULTS

To determine whether MCF-7 breast cancer cells that express HER2 produced Ang-2, a Northern blot analysis of total RNA extracted from MCF-7 cells was performed with the use of a specific probe for Ang-2. Human placenta was used as a positive control. Total RNA from normal human breast tissue was also probed for Ang-2 mRNA production. Ang-2 mRNA was detected in MCF-7 cells and in placenta but undetectable in normal human breast tissue (Fig 1). To determine whether HER2 signaling influences regulatory control of Ang-2 mRNA production, the MCF-7 cells were pretreated for 24 hours with either Herceptin or Heregulin β1 to block or stimulate, respectively, HER2 dimerization

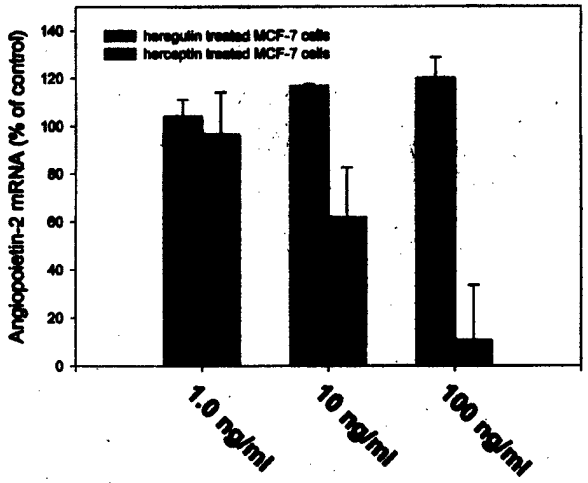

Fig 2. Dose response of Ang-2 mRNA expression to heregulin β1 and Herceptin treatment. Total RNA (2 μg) from MCF-7 cells treated with either Herceptin or Heregulin β1 for 24 hours. Ang-2 mRNA was determined by semiquantitative RT-PCR. Significance was achieved at 100 ng/mL of Heregulin β1 ($P < .05$) and 10 ng/mL Herceptin ($P < .01$). Product from RT-PCR was normalized to concurrent β-actin mRNA RT-PCR, and statistical analysis was performed after normalization was achieved as a percent of control.

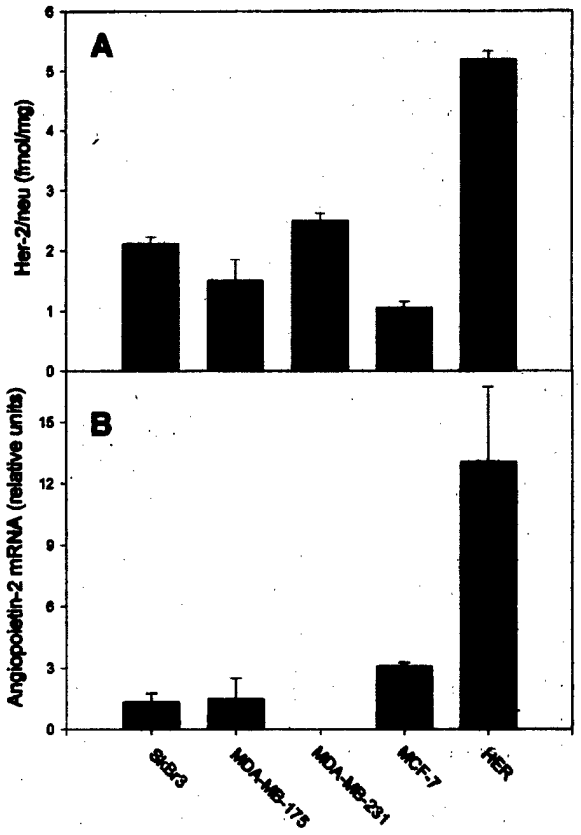

Fig 3. A, Expression of HER2 (femtomole/milligram) in breast cancer cell lines, determined by ELISA. **B,** Ang-2 expression in human breast cancer cell lines by RT-PCR with specific primers for Ang-2 (relative units determined by normalization to concurrent β-actin mRNA RT-PCR).

and signaling. Herceptin blockade of HER2 signaling (100 ng/mL) reduced Ang-2 mRNA production (Fig 1). Conversely, stimulation of HER2 signaling with Heregulin β1 (1.0 ng/mL) upregulated Ang-2 mRNA (Fig 1).

Using RT-PCR with specific primers for Ang-2, we tested pretreated MCF-7 cells for the production of Ang-2. Using 3 doses of Herceptin (1.0, 10, and 100 ng/mL) and Heregulin β1 (1.0, 10, and 100 ng/mL), we constructed dose-response curves for Ang-2 production (Fig 2). The Herceptin-treated cell lines showed a marked decrease in Ang-2 mRNA production, determined by densitometry normalized to concurrent β-actin RT-PCR. A 90% reduction in Ang-2 mRNA was seen in MCF-7 cells treated with 100 ng/mL of Herceptin ($P < .001$ vs untreated cells). Pretreatment with Heregulin β1 dose-dependently increased Ang-2 mRNA production (Fig 2). At 100 ng/mL of Heregulin β1, there was a 20% increase in Ang-2 mRNA compared with control cells ($P < .05$).

To determine whether HER2 expression is linked to Ang-2 mRNA production in other breast cancer cell lines, we performed RT-PCR for Ang-2 using SK-BR3, MDA-MB-175, and MDA-MB-231 cell lines. We also tested HER cells, a stably transfected cell line derived from MCF-7 cells that overexpress HER2 6-fold. Fig 3 shows the relative expression of HER2 protein and Ang-2 mRNA in the cell lines. All of these cell lines expressed HER2 protein (Fig 3, *A*), and all but MDA-MB-231 expressed Ang-2 mRNA.

Lastly, we sought to determine whether Ang-2 mRNA production was found in human breast cancers that express HER2. Eleven archived breast cancer specimens and 3 normal breast epithelium specimens were assessed for HER2 protein by ELISA and for Ang-2 mRNA by RT-PCR. Five of 11 human breast cancers expressed HER2 (Fig 4, *A*), and 7 of 11 breast cancer tissues produced Ang-2 mRNA (Fig 4, *B*). All 5 HER2 expressors produced Ang-2 mRNA. There was no HER2 expression or Ang-2 mRNA detected in normal human breast epithelium.

DISCUSSION

HER2 overexpression is associated with a poor clinical prognosis in which patients have shorter disease-free and overall survival.[1-3] These findings suggest that HER2 expression is linked to downstream changes in gene expression, which enhance

the cells' metastatic ability. In this report, we sought to establish a link between HER2 expression and Ang-2 production, a putative vascular destabilizing factor. In previous work, we have determined that the enhanced metastatic phenotype of breast cancer cells that express HER2 appeared to be at least partly due to transendothelial migration of the tumor cells. The mechanism of transmigration appears to be the induction of EC retraction, with loss of EC-cell contact and barrier function. Because of the vascular destabilizing influence of cancer cells that are stimulated by HER2 signaling, we postulated that HER2 induces upregulation of Ang-2 with the profound effects on the endothelium noted. Blockade of HER2 signaling with the use of Herceptin reduced Ang-2 mRNA production by 90% in our study, although stimulation of HER2 signaling increased Ang-2 expression. These data suggest that HER2 signaling influences Ang-2 mRNA production, which supports our hypothesis. It is possible that Ang-2 production may be a mechanism of HER2-induced metastatic capability. Alternately, HER2 influence on cell proliferation or cell cycle regulation may be at least partly responsible for the alteration in Ang-2 expression. Further studies are necessary to demonstrate a direct effect of HER2 signaling on Ang-2 transcription and to identify which transcription factors may mediate the effect.

We have shown that MCF-7 cells, which express normal quantities of HER2 (1.0 fmol/mg tissue), produced Ang-2 mRNA. MCF-7 cells are considered to have low metastatic capability and in vitro induce a modest EC retraction response on endothelial monolayers (unpublished data). The MCF-7 transfected cell line (HER cells that overexpress HER2) show a much greater in vitro metastatic aggressiveness and a profound increase in Ang-2 mRNA. Antibody blockade of HER2 signaling has significantly reduced the aggressive phenotype of HER2 that expresses breast cancers both in vivo[13,14] and in vitro and reflect the changes seen in Ang-2 expression in this study. Oh et al[15] also identified several genes with expression levels that were altered in MCF-7 cells when modified by transfection to overexpress HER2. We further studied other breast cancer cell lines that overexpress HER2 to apply this hypothesis outside the MCF-7 model. We identified Ang-2 production in 2 of 3 of these cell lines. These studies indicate that HER2 signaling may influence alteration of downstream gene expression, specifically Ang-2.

The overexpression of HER2 is found in 25% to 30% of breast cancers. In the specimens tested for this study, 5 of 11 cancers expressed HER2, and all

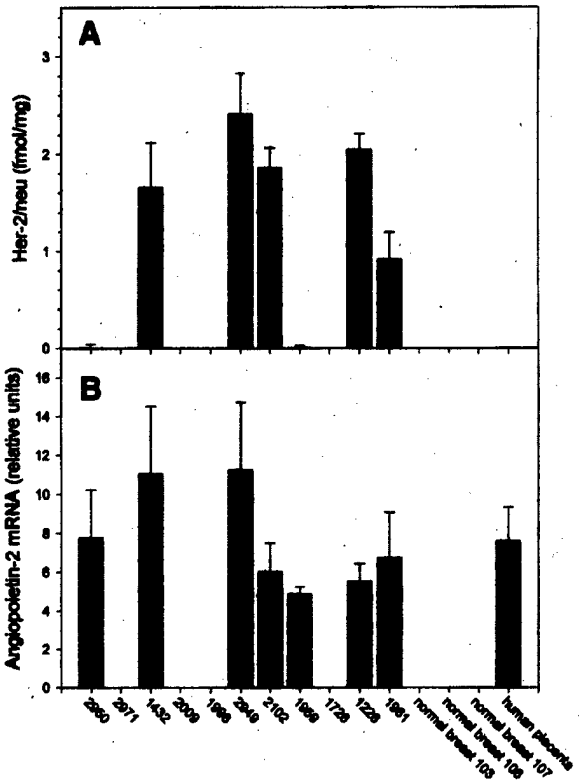

Fig 4. A, Expression of HER2 (femtomole/milligram) in human breast cancer specimens, determined by ELISA. **B**, Ang-2 expression in human breast cancer specimens and normal breast epithelium by RT-PCR with specific primers for Ang-2 (relative units determined by normalization to concurrent β–actin mRNA RT-PCR). Human placenta RNA was used as a positive control for Ang-2 expression.

5 produced Ang-2 mRNA. Two additional cancers had no detectable HER2 expression by ELISA yet produced Ang-2 mRNA. Further, 1 breast cancer cell line, MDA-MB-231 cells, expressed HER2 but had no detectable Ang-2 mRNA production. These data support the HER2 signaling link to Ang-2 production but clearly illustrate that HER2 expression and signaling are not the only potential pathways to induce Ang-2 mRNA. It is possible that other factors that alter cell growth independent of HER2 may induce Ang-2 expression.

Published data suggest that relative dimerization patterns of HER2 with other members of the type I growth factor receptor family can influence particular cell signaling pathways stimulated by HER2 signaling.[20] This compartmentalization of signaling can support a variety of transcription pathways. Using ovarian cell lines, Xu et al[21] showed that the level of HER2 expression, relative to HER3 and HER4, could modulate the response to heregulin, thereby determining whether a response is stimulatory or inhibitory. It is possible that other dimer-

ization patterns of these type I receptors, perhaps involving EGFR, can also induce a signaling pathway leading to Ang-2 production as seen in 2 of the breast cancer specimens. It is also possible that the compartmentalization of the signaling in the MDA-MB-231 cells bypasses Ang-2 mRNA transcription. Alternatively, the HER2 receptor expressed in these cells may not have an intact signaling mechanism. DiGiovanna et al[23] showed that the expression of HER2 does not necessarily imply signaling. In that study, only 35% of HER2-overexpressing breast cancers actively signaled through the HER2 receptor. Further delineation of the relative expression of all these receptors and the signaling transduction pathways in Ang-2 producing breast cancers would help to understand this mechanistic pathway.

In this report, we have identified Ang-2 production in human breast cancer cells that express HER2 and further linked the regulation of Ang-2 production in these cells to HER2 signaling. We also identified Ang-2 mRNA in human breast cancer specimens and provided evidence of a link between HER2 expression and Ang-2 in these tumors. The findings of HER2 signaling–linked Ang-2 production makes Ang-2 and its receptor, Tie-2, potential therapeutic targets for the treatment of HER2 that expresses breast carcinoma, which may act synergistically with Herceptin. Further, the expression of Ang-2 may define which patients may benefit from Herceptin therapy.

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HER2/NEU OVER-EXPRESSION INDUCES ENDOTHELIAL CELL RETRACTION

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Over-expression of the *HER2/neu (HER2)* proto-oncogene in breast carcinoma imparts an enhanced metastatic potential. Metastasis requires escape of the tumor cell from the vasculature into subjacent tissue, a transmigration event across an endothelial cell (EC) monolayer. EC retraction has been reported to precede transmigration in several tumor metastatic models. Using intact human iliac vein EC monolayers, we tested the abilities of MCF-7 breast cancer cells and HER cells, a transfected MCF-7 line over-expressing *HER2*, to induce EC retraction. We further analyzed whether *HER2* signaling influenced cancer cell–induced EC retraction. MCF-7 or HER cells were co-cultured onto mature EC monolayers. More HER than MCF-7 cells induced EC retraction ($76 \pm 19\%$ *vs.* $17 \pm 12\%$, $p < 0.001$) with resultant exposure of subendothelial matrix ($6.80 \pm 2.86\%$ *vs.* $0.85 \pm 0.39\%$, $p < 0.001$). Blockade of *HER2* signaling using Herceptin™ nearly eliminated EC retraction ($p < 0.01$), while stimulation of *HER2* using heregulin-β1-augmented EC retraction ($p < 0.05$). Further, there was no difference between cell lines in either the number of cells adhered or the strength of adherence to EC under shear stress. These data suggest that *HER2* signaling enhances metastasis in breast cancer cells by inducing EC retraction, a process that appears to precede endothelial transmigration.

Key words: HER2/neu *over-expression; breast carcinoma; metastasis*

Breast cancer strikes 1 in 9 women and is a major cause of cancer-related death.[1] Significant advances have been made in the early detection and surgical treatment of breast cancer, but there has been only negligible impact on the mortality rate. To improve outcome, better treatment of metastatic disease is important. One promising strategy is to arrest metastases at key steps in the metastatic process.

Transcellular migration of tumor cells across the endothelium is an essential step in metastasis. This egress into secondary tumor sites is likely a rate-limiting step in the development of metastases, yet the mechanisms of this process are largely unknown. Intact endothelium provides some barrier to resist tumor cell invasion,[2] yet tumor-cell interactions involving the basement membrane, extracellular matrix and endothelial cells (ECs) of the microvasculature often overcome these defenses. Intercellular junctions are targeted sites for this transcellular process, possibly through EC retraction with exposure and degradation of matrix molecules and basement membrane.[3–5]

The ability of tumor cells to metastasize is multifactorial. Expression of certain genetic or phenotypic characteristics is associated with greater metastatic potential and poor prognosis. In breast cancer, over-expression of the *HER2* (c-*erbB2*) proto-oncogene has such an association. *HER2* is over-expressed in 30% of breast cancers and is the most powerful predictor of disease-free and overall survival rates after lymph node status.[6–8] *HER2*, a type 1 growth factor receptor expressed inversely with estrogen receptor, is a tyrosine receptor kinase. Tyrosine kinase receptors are expressed in a wide range of different cell types and are mediators of cell growth, differentiation and survival. Four distinct members of this family have been identified: *EGFR, HER2, HER3* (c-*erbB3*) and *HER4* (c-*erbB4*). A class of ligands, the neuregulins, bind to *HER3* or *HER4*, causing heterodimerization with *HER2*.[9,10] *HER2* is the signaling subunit and has no independent ligand. Antibody blockade of *HER2*, preventing heterodimerization, eliminates neuregulin-stimulated signaling.[11–14] Prior investigations have shown *HER2* to be an independent prognostic indicator in breast,[15] ovarian[16] and lung[17] carcinomas, which have associated early metastases. *In vitro* studies show that cancer cells with increased expression of *HER2* have more aggressive patterns and greater numbers of metastatic sites.[6,8,18] Antibody blockade of *HER2* receptors restricting *HER2*-mediated cell signaling may improve tumor regression and delay return to a tumor growth phase in patients with aggressive breast cancer.[18,19] The potential mechanism of enhanced metastatic capability, however, remains speculative.

In this study, we sought to determine if over-expression of *HER2* enhances breast cancer cell–induced EC retraction. Using *HER2*–over-expressing MCF-7 cells, which are constitutively active as a common pathway of neuregulin signaling, we evaluated the ability to stimulate EC retraction as a mechanism of transmigration and whether *HER2* signaling regulated tumor cell–induced EC retraction.

MATERIAL AND METHODS

EC culture

Human adult ECs were isolated from iliac veins and cultured according to the methods of Jarrell *et al.*[20] Briefly, iliac vein segments from heart-beating, brain-dead cadavers were treated with collagenase to release lumenal endothelium. Cells were plated and passed on gelatin-coated tissue plastic in medium M-199 supplemented with 15% FBS, EC growth factor supplement and heparin. EC monolayer cultures with barrier function were established by plating low-passage cells at 90% density and growing them to confluence. These cells were allowed to establish stable monolayers over 5 days prior to retraction experiments.

Breast-cancer cells

MCF-7 (ATCC, Rockville, MD) and HER cell lines, a stably transfected MCF-7 cell line over-expressing *HER2*,[21] were established in our laboratory. They were maintained in high-glucose DMEM supplemented with 10% FBS and penicillin (1,000 U/ml)/streptomycin (1 mg/ml). HER cells were additionally supplemented with G418 (300 μg/ml) to select only expressing transfected cells. Low-passage cells were labeled and used for transmigration experiments. Although the basal level of expression in the MCF-7 line is likely susceptible to up-regulation in estradiol-starved colonies, no change in expression was seen throughout the course of our study.

EC retraction assay

MCF-7 or HER cells in human complete medium were co-cultured at 2×10^5 cells/cm^2 onto 5-day-old, mature, confluent monolayers of HIVE cells. After 3 or 12 hr of co-culture, cells were fixed with 2.3% paraformaldehyde and prepared for SEM or

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digital microscopy imaging. Other tumor cells in 12 hr experiments were also pre-treated with either 100 ng/ml Herceptin (gift of Genentech, South San Francisco, CA) to block *HER2* signaling or 1.0 ng/ml heregulin-β1 to stimulate *HER2* signaling. After fixation, 3 hr co-cultures were washed in DCF-PBS with 25 mM PIPES. A disc of 6 mm diameter was cut from the culture dish and ethanol-extracted in series from 50% to 100% ethanol. The disc was then critical point-dried, mounted and sputter-coated with gold. Five random SEM images were obtained digitally using a Jeol (Peabody, MA) JS850 scanning electron microscope (magnification ×500). EC retraction was determined both by counting the number of tumor cells associated with an EC retraction event and by calculating the percent area of subendothelial matrix exposed by the retracting ECs using image analysis software. Twelve-hour EC retraction assays were calculated after digitally capturing 5 contiguous images using a 10× objective with phase-contrast microscopy, then calculating the percent area exposed by retraction using enhanced image analysis (SigmaScan Pro; Jandel, Corte Madera, CA). For statistical analyses, Student's *t*-test was used between groups.

Radial flow detachment assay

This novel adhesion assay was used to evaluate cell–cell adhesion under shear forces experienced by ECs *in vivo*. The assay was modified from DiMilla *et al.*[22] In brief, HIVEC confluent monolayers were maintained in 60 mm tissue culture dishes for 5 days to allow development of mature adherens junctions. MCF-7 or HER cells were labeled with the intravital nuclear dye bis-benzimide (BBI), and plated onto confluent HIVEC monolayers at 1×10^5 cells/mm^2. After 1 hr of co-culture, cells were exposed to shear force in the radial flow chamber. Tissue culture medium containing 15% FBS was applied at the center point of the flow chamber at 3 ml/sec, creating a fluid axisymmetric shear, in which the hydrodynamic shear force on the cell (s, μdyn/μm^2) decreases with the radial distance (r, mm) from the central inlet point

$$s = \frac{1.5Q}{r - 1.7}$$

and Q represents the volumetric flow rate (ml/sec). During this shear flow, cells detach from the inner region, where shear is greater than attachment strength. As the shear decreases with increasing distance from the central infusion point, a greater number of cells remain attached. After 5 min of flow, cells were fixed and counted mechanically at 1 mm intervals. Data were plotted as cell number *vs.* distance from center, reflecting shear, and interpreted by comparing the r_{50} (radius at which 50% of the maximal number of cells are detached) using Student's *t*-test.

RESULTS

To evaluate the role of *HER2* activity in cancer-cell function, we compared MCF-7 breast-cancer cells and MCF-7 cells over-expressing a constitutively active form of *HER2* (HER cells). These cells were evaluated for expression using a commercial ELISA kit (Sigma, St. Louis, MO). HER cells (5.28 fmol/mg) express more than 6 times more *HER2* than MCF-7 cells (0.80 fmol/mg).

The mechanism of metastasis involves tumor cell–EC interaction and transmigration of the tumor cell across the EC. To test the possibility that *HER2* activity influences EC retraction as a key step in tumor cell transmigration, we tested MCF-7 cells and HER cells for the ability to induce EC retraction of 5-day intact HIVE monolayers. Tumor cells (1×10^5/cm^3) were placed in direct co-culture with the HIVE monolayers. EC monolayers were intact with a typical cobblestone appearance at the beginning of all experiments. Tumor cells were noted to adhere to the monolayer overlying the intercellular junctions. At 3 hr in co-culture, monolayer integrity was lost at specific focal areas corresponding with tumor-cell adherence to the EC. At these sites, individual ECs appeared to retract, opening a portal for transmigration of tumor cells. Experiments were fixed at 3 hr and evaluated by SEM. SEM images of an EC monolayer co-cultured with MCF-7 (Fig. 1*a*) or HER (Fig. 1*b*) cells are shown. These images demonstrate areas of intact EC monolayer and retraction of individual ECs in proximity to HER cells. MCF-7 cells show only small areas of retraction immediately adjacent to the tumor cells. Tumor cells associated with a retraction event were counted and compared to control monolayers. HER cells were associated with significantly more retraction events than MCF-7 cells at 3 hr (76 ± 19% *vs.* 17 ± 12%, $p < 0.001$; Fig. 2*a*). Retraction events were also evaluated by calculating the percent area of subendothelial matrix that was exposed by EC retraction. The subendothelial matrix exposed by HER cell–stimulated EC retraction was 6.80 ± 2.86% of the total area. MCF-7 cell–stimulated subendothelial matrix exposure was 0.85 ± 0.39% ($p < 0.001$). Control monolayers of ECs alone had 0.16 ± 0.14% of the subendothelial cell matrix exposed (Fig. 2*b*).

To determine if HER2 signaling in these cancer cells induced EC retraction, we treated MCF-7 and HER cells for 24 hr with Herceptin™ (100 ng/ml), to block *HER2* dimerization and signaling, or heregulin-β1 (1.0 ng/ml), to stimulate dimerization and *HER2* signaling. Figure 3 shows MCF-7 cells in co-culture with HIVE monolayers at 12 hr. Significant EC retraction and matrix exposure were distinctly identified in the co-culture experiments (Fig. 3*b*), while typical cobblestone architecture was seen in control monolayers (Fig. 3*a*). When Herceptin™ blocked *HER2* signaling, the monolayer remained nearly intact at 12 hr (Fig. 3*c, cf.* Fig. 1*b* at 3 hr). Conversely, treatment with heregulin-β1 to stimulate *HER2* signaling induced a marked increase in the architectural breakdown of the EC monolayer (Fig. 3*d*). In both cell lines, pre-treatment with heregulin-β1 induced greater EC retraction ($p < 0.05$, Fig. 4). Pre-treatment with Herceptin™ significantly decreased EC retraction in both cell lines, nearly eliminating the effect in MCF-7 cells (17.23 ± 3.6% *vs.* 2.88 ± 1.1%, $p < 0.01$, Fig. 4).

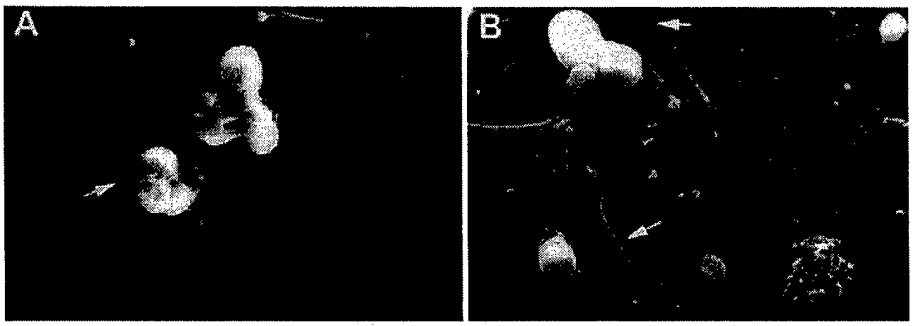

FIGURE 1 – SEM images (×500) of MCF-7 *(a)* and HER *(b)* cells at 3 hr of co-culture with intact HIVE monolayers. MCF-7 cells induced limited EC retraction immediately adjacent to the tumor cells, while HER cells induced more extensive EC retraction. Arrows indicate retracting ECs.

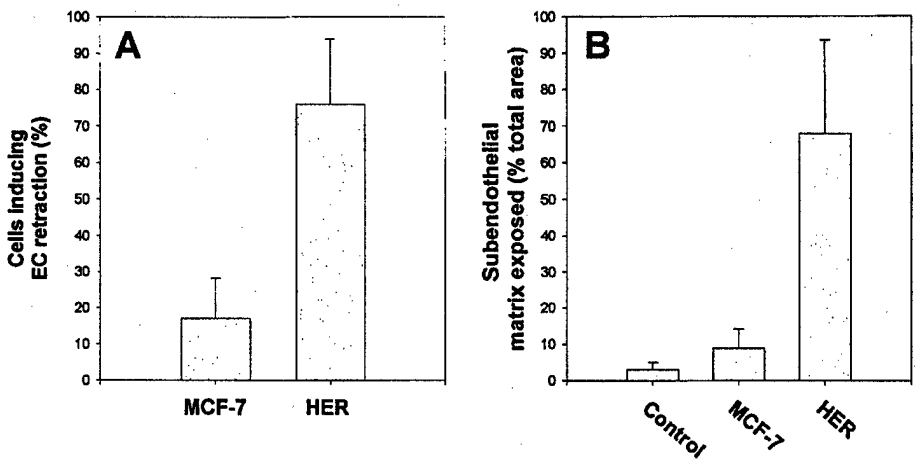

FIGURE 2 – *(a)* Number of tumor cells associated with an EC retraction event, counted using SEM images (×500) at 3 hr of co-culture with HIVE monolayers. *(b)* Degree of subendothelial matrix exposed by retracting ECs as a percent of total area. Matrix exposure was calculated by image analysis software at 3 hr of co-culture, n = 5, $p < 0.001$, MCF-7 *vs.* HER.

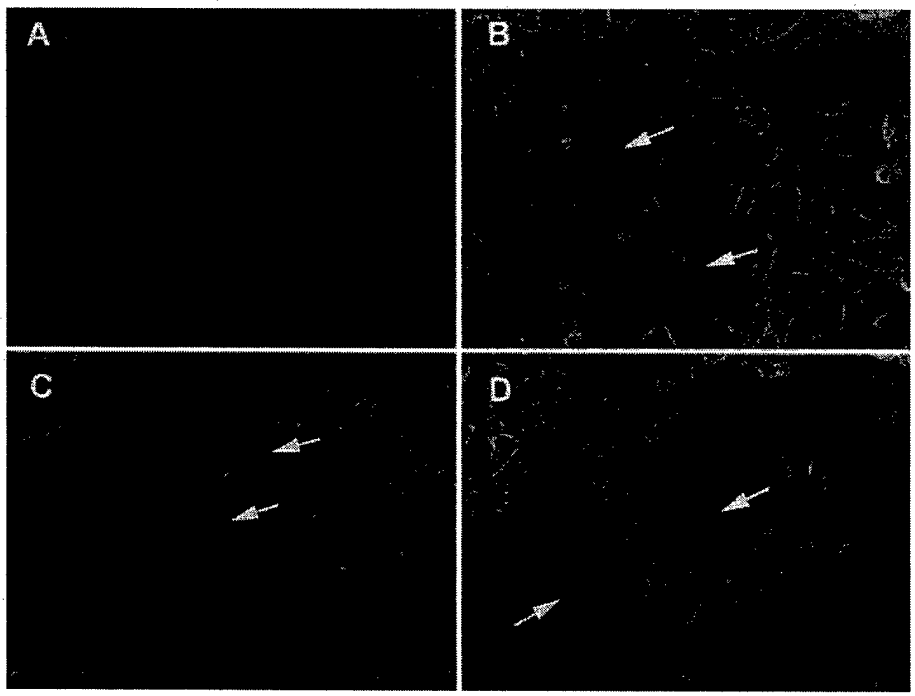

FIGURE 3 – Enhanced phase-contrast images of intact HIVE monolayers at 12 hr of co-culture with MCF-7 cells. *(a)* Control HIVE monolayer. *(b)* MCF-7 cells in co-culture. Arrows indicate subendothelial matrix exposed after EC retraction. *(c)* Herceptin® (100 ng/ml)–treated MCF-7 cells in co-culture. Arrows identify tumor cells. *(d)* Heregulin-β1 (1.0 ng/ml)–treated MCF-7 cells in co-culture. Arrows indicate subendothelial matrix exposed after EC retraction.

Alteration of tumor-cell adherence may influence EC retraction events. To determine if *HER2* over-expression alters tumor-cell adherence, we evaluated both the number of tumor cells adherent to the monolayer and the strength of tumor cell–EC adherence. We hand-counted the adhered tumor cells. There was no difference in the number of MCF-7 cells adhered to the EC monolayer compared to HER cells (31.58 ± 7.17 *vs.* 35.12 ± 6.14 cells/mm^2, p = 0.18). Further, we determined the strength of tumor-cell adherence to the EC monolayer using a radial flow detachment assay. This assay reproduces the intralumenal shear forces normally encountered by the EC environment. After 1 hr of co-culture of tumor cells on EC monolayers to allow adherence, cells were exposed to radial flow of 3 ml/sec. After 5 min of shear force exposure, r_{50} was calculated. There was no difference in the strength of adherence between MCF-7 and HER cells (2.91± 0.91 *vs.* 4.02 ± 1.93, p = 0.22).

DISCUSSION

For these experiments, we used MCF-7 human breast cancer cells, which minimally express *HER2* and are considered to be well-differentiated with limited metastatic potential. We tested these cells against a stably transfected cell line derived from MCF-7 cells that constitutively over-expresses *HER2,* to demon-

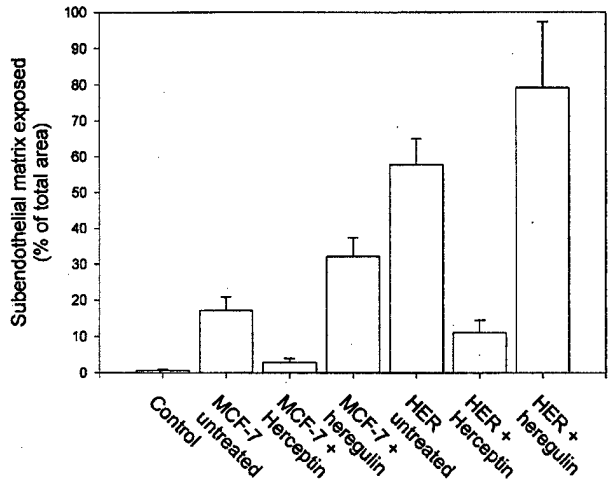

FIGURE 4 – Degree of subendothelial matrix exposed by EC retraction as a percent of total area. Matrix exposure was calculated by image analysis software of images captured digitally after 12 hr of co-culture. Tumor cells were treated with 100 ng/ml Herceptin or 1.0 ng/ml heregulin-β1 in culture medium or medium alone. All co-culture experiments were different from control HIVE monolayers ($p < 0.05$). Herceptin™ treatment blocked EC retraction ($p < 0.01$ *vs.* MCF-7, $p < 0.001$ *vs.* HER). Heregulin-β1 treatment increased EC retraction ($p < 0.05$ *vs.* MCF-7 and HER).

strate a potential role of *HER2* over-expression in the metastatic process. Tumor cell–induced EC retraction was influenced by *HER2* signaling. Over-expressing HER cells induced significantly more EC retraction events with subendothelial matrix exposure.

Several studies, both *in vitro* and *in vivo,* have shown that ECs retract prior to tumor-cell extravasation. Nicolson[23] reported that melanoma cells induce EC retraction, allowing a portal of transmigration. This EC retraction has also been reported in pancreatic,[24] lung[25] and breast[26,27] cancer cells. Tumor cells appear to preferentially adhere to ECs at the inter-EC junctions. After tumor-cell attachment, EC retraction is initiated and tumor cells spread on the exposed subendothelial matrix. *HER2* signaling clearly enhanced the cellular ability to stimulate EC retraction. These data imply that EC retraction may be a key step in the transmigration of breast cancer cells and that this process is driven by *HER2* signaling. The specific products resulting from *HER2* signaling that induce EC retraction remain to be determined.

Endothelial injury has been linked to retraction. EC injury due to oxidative stress induced by H_2O_2 or gamma irradiation[28] stimulated EC retraction. These processes were reversible upon removal of the noxious stimuli. EC injury also increases adhesion of tumor cells to endothelium. EC injury does not appear to be the mechanism of EC retraction in our model. EC retraction was identified only in association with tumor cells, and the ECs remained viable by Trypan blue exclusion. Further, no difference was seen between the cell lines in the strength of adhesion to Ecs, as would be expected in an EC injury model.

Conditioned medium from MCF-7 cells also induces EC retraction.[24,27] The putative factor is heat-stable, m.w. 10 to 50 kDa. The factor binds to heparin-Sepharose and is degraded by trypsin. Inhibition of protein synthesis reduced EC retraction by 50%.[24] These data suggest that in MCF-7 cells EC retraction is dependent on a secreted protein product. We have also identified EC retraction in Transwell co-cultures of MCF-7 cells with EC, consistent with this report (data not shown.) Lewalle *et al.*,[26] however, reported EC retraction only with direct cell–cell contact. This apparent discrepancy may be resolvable in that their report evaluated only much shorter contact times than the prior report. It is possible that a secreted product reaches a significant concentration only in close proximity during early co-culture and that longer exposure times induce a broader effect. Lewalle *et al.*[26] also demonstrated that MCF-7–induced EC retraction is linked to loss of VE-cadherin expression at the site of retraction, with profound changes in vinculin distribution.

The prior reports on MCF-7–induced EC retraction did not discuss the possible role of *HER2.* Our data strongly suggest that *HER2* signaling can induce EC retraction, serving as a portal for transmigration. Yoshioka *et al.*[27] reported that MCF-7–induced EC retraction is reduced by pre-treatment with estrogen/progesterone. Pre-treatment with estrogen down-regulates *HER2* in MCF-7 cells, which is a plausible explanation of these data. Yu *et al.*[25] lend further support for the link of *HER2*-stimulated transmigration: stable transfection of $p185^{neu}$, the signaling subunit of *HER2,* into non-small-cell lung-carcinoma cells increased pulmonary and extrapulmonary metastases when introduced intravascularly. A549 lung-carcinoma cells, which express *HER2,* also stimulate EC retraction.[3]

Over-expression of a cellular molecule may facilitate malignancy and drive the metastatic process. A shift in breast cells from low *HER2* expression to higher expression not only results in different responses to estrogen by the tumor cell but also affects the local vasculature in a way that permits metastasis. Our results suggest that *HER2* signaling induces a breakdown of the vascular barrier, which may be sufficient to permit tumor-cell transmigration and metastasis.

In conclusion, we have demonstrated that a step in the metastatic cascade, tumor-induced EC retraction, is enhanced by *HER2* signaling. *HER2* over-expression did not appear to alter tumor-cell adhesion or strength of adhesion to ECs. Future studies will be directed at delineating the molecular mechanism of *HER2*-induced EC retraction.

ACKNOWLEDGEMENTS

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HER2 signaling–induced microvessel dismantling

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Background. *The human epidermal growth factor receptor 2 protein (HER2) signaling in breast cancer imparts a metastatic advantage to the cell, likely by regulating gene expression. The HER2 signaling upregulates angiopoietin-2 (Ang-2), which disrupts endothelial cell (EC) adherens junctions. We postulated that HER2 signaling may facilitate angioinvasion by disrupting microvessel integrity.*
Methods. *Rat microvessels, embedded in collagen, were grown into capillary networks and cocultured with MCF-7 or HER2 overexpressing MCF-7 (HER) to test for microvessel breakdown. We quantitated this effect by determining the cumulative length of intact microvessels. Other experiments used Herceptin- or heregulin β1-pretreated MCF-7 cells to modulate HER2 signaling, or soluble Tie-2/Fc receptor fusion protein (sTie2) to sequester tumor-cell released Ang-2.*
Results. *The MCF-7 cells induced a time-dependent loss of microvessel integrity. At 12 hours, HER cells induced a 90% reduction in cumulative length ($P < .05$). Pretreatment with Herceptin reduced whereas heregulin β1 augmented microvessel dismantling ($P < .01$). Sequestration of Ang-2 significantly, though not dramatically, reduced the MCF-7 cell induction of microvessel dismantling ($P < .01$).*
Conclusions. *We show that HER2 signaling in breast cancer cells leads to induction of microvessel dismantling, which may open a portal for angioinvasion. It appears that Ang-2 affects this mechanism, although other factors also function in microvessel dismantling. (Surgery 2001;130:382-7.)*

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THE OVEREXPRESSION OF HER2 (neu, c-erbB2) imparts an aggressive metastatic phenotype in breast cancer. Second only to lymph node status, HER2 protein overexpression is a powerful predictor of whether the patient is disease free and of the survival prognosis.[1-3] A member of the epidermal growth factor receptor family, HER2 is expressed in a range of cell types and functions in growth, differentiation, and survival.[4,5] The ligand class for these receptors is the neuregulin, which is relatively specific in its binding. Neuregulin binding induces heterodimerization with HER2, which is the signaling subunit and has no independent ligand.[6,7] The relative dimerization patterns of HER2 with other receptors influences specific cell signaling pathways.[8] This signaling compartmentalization can support a variety of transcription pathways, with divergent downstream events. The dimerization pattern of HER2 with HER3 relative to HER4 can modulate the response to heregulin, inducing a response that is either stimulatory or inhibitory.[9] Antibody blockade of HER2, which prevents heterodimerization, can eliminate neuregulin-stimulated signaling.[10-13] Signal down modulation can induce tumor regression in breast cancer cells expressing HER2.[14,15]

The metastatic advantage imparted to cancer cells by over-expressing HER2 has not been clearly delineated. In testing the hypothesis that HER2 signaling is linked to cellular production of factors that influences the metastatic phenotype, we have shown that breast cancer cells expressing HER2 can induce endothelial cells (ECs) to retract, creating a gap in the endothelial monolayer.[16] This gap may serve as a portal for tumor cell transendothelial migration, a key step in the metastatic process. This ability of the cell to induce EC retraction can be regulated by modulation of HER2 signaling by using monoclonal antibody blockade of HER2 dimerization or signal induction with heregulin β1 stimulation. Oh et al[17] also have identified downstream alterations in gene expression as a direct consequence of HER2 signaling.

Because of its profound effects on ECs, we have

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Fig 1. Dismantling of microvessel networks by coculture with HER cells. **(A)** Control vessels after 8 days in collagen I gel with DMEM + 10% FBS. **(B)** Microvessels after 12 hours of coculture with 1×10^3 HER cells/mm^2. *Arrows* indicate regions of dismantling and microvessel discontinuity. Images are phase contrast at 4 × and embossed to enhance detail.

postulated that Ang-2 may be a factor involved in HER2-induced EC retraction. Ang-2 is a key protein involved in angiogenesis, the development of neovessels from existing blood vessels. Angiogenesis involves EC dissociation, shape change, and invasion of the supporting matrix.[18] The EC specific tyrosine kinase receptors, Tie-1 and Tie-2 (tek), appear to be key signaling elements involved in angiogenesis. The ligands for Tie-2, angiopoietin-1 (Ang-1), and Ang-2 are antagonistic although they bind with equal affinity.[19] The Ang-1 appears to function in the stabilization of vessels, whereas Ang-2 binding to Tie-2 results in destabilization of the vessel with dissociation of EC-EC junctions.

We have shown that HER2 signaling induces EC retraction. The mechanism appears to be the induction of dissociation of α, β, and γ catenin from VE cadherin, breaking the link between the adherens junction and the cytoskeleton.[20] We also have shown that HER2 signaling appears to regulate the cellular production of Ang-2 in MCF-7 cells,[21] and we have linked Ang-2 production to EC retraction and the catenin:cadherin dissociation.

These findings led us to hypothesize that HER2 signaling-induced production of Ang-2 in breast cancer cells would initiate vascular disassembly, imitating the angiogenic regression phase. This architectural dismantling of microvessels potentially would open a portal for angioinvasion of these phenotypic cells, a key step in the metastatic process.

In this study, we tested MCF-7 cells and HER2 overexpressing MCF-7 cells for the ability to induce microvessel dismantling of an in vitro microvessel network. Further, we manipulated HER2 signaling by using Herceptin and heregulin β1 to test for HER2 regulation of microvessel dismantling. Finally, we treated MCF-7 cells with Tie-2/Fc soluble receptor fusion protein to quench cell-released Ang-2 and tested for microvessel dismantling.

MATERIAL AND METHODS

Cell lines. The MCF-7, SK-Br3, and MDA-MB-175 cells (American Type Culture Collection) and HER cells, a stable transfected MCF-7 cell line overexpressing HER2 6-fold (gift of C. Benz, San Francisco, Calif[22]) were maintained in high glucose, Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (1000 U/mL) and streptomycin (1 mg/mL), at 37°C, 5% CO_2. The HER cells also were supplemented with G418 (300 μg/mL) to maintain selection of expressing, transfected cells.

Microvessel dismantling assay. Epididymal fat pads were harvested from male 200-g Sprague-Dawley rats. Approximately 10 g of fat were minced and digested with a 0.25% collagenase III (Worthington Biochemical Corp, Lakewood, NJ) solution for 5 minutes. The tissue was rinsed in phosphate-buffered saline solution + 0.2% bovine serum albumin, and sequentially filtered by using mesh sizes of 350 μm and 30 μm to isolate microvessel fragments from undigested tissue and subsequently from cells and debris. Eight to 10,000 vessels/mL were suspended in a matrix of collagen I (3 mg/mL) in DMEM and incubated in humidified 37°C and 5% CO_2, with DMEM + 10% fetal bovine serum in 24-well tissue culture plates. Vessels remained in culture for 12 days to allow for capillary network development. Testing of cancer cell lines occurred on day 12. Cells were harvested from log-phase growth cultures and added to wells at 1×10^3

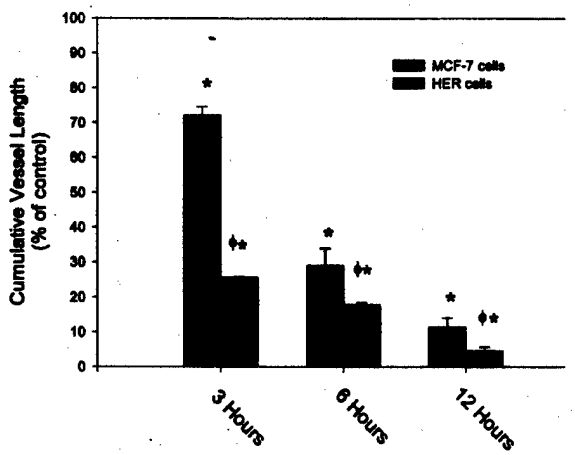

Fig 2. The HER2 induced microvessel dismantling. The MCF-7 or HER cells are cocultured at 1×10^3 cells/mm^2 with 12-day-old microvessels embedded in collagen I gel. Cumulative microvessel length was determined and compared with control (untreated microvessel gels.) The HER cells cause a more rapid and greater extent of dismantling compared with parental MCF-7 cells ($P < .05$ for all time points between cell types, and *asterisk*, $P < .01$ for both cell lines compared with control, n = 12 MCF-7; n = 3 HER).

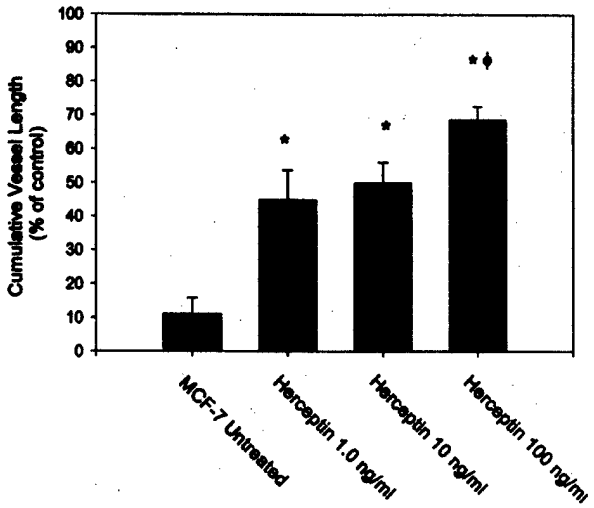

Fig 3. HER2 signaling blockade inhibited microvessel dismantling. MCF-7 cells were pretreated with increasing doses of Herceptin for 24 hours before coculture with microvessel collagen I gels for 12 hours. Cumulative microvessel length was determined and compared with control. Treated cells were dose-dependently inhibited from inducing dismantling (*asterisk*, $P < .01$ vs untreated MCF-7 cells; $P < .01$ vs 1.0 ng/mL; n = 3).

cells/mm^2. For HER2 signaling experiments, MCF-7 cells were pretreated with 1 or 100 ng/mL Herceptin (gift of Genentech, San Francisco, Calif), or 0.1 or 1.0 ng/mL heregulin β1 (Genentech) for 24 hours before the microvessel-dismantling assay. In other experiments, 2, 20, or 200 ng/mL of sTie2 was added with MCF-7 cells or alone to the microvessel cocultures. After 3 to 12 hours of coculture, the gels were fixed with 3.2% paraformaldehyde, and stained with rhodamine-conjugated Griffonia simplificolia-1 (Sigma Chemical Co, St Louis, Mo), a lectin that specifically intercalates into rat endothelium. The gels then were sandwiched between glass slides and imaged by using an Olympus BH-2 microscope equipped with a Pixera digital camera (Pixera Corp). Microvessel length was calculated by linear tracing of the stained microvessels, minus the parent vessels, by using SigmaScan Pro image analysis software. Statistical analysis was performed by Student *t* test and analysis of variance between groups.

RESULTS

Rat microvessels in collagen I gels treated with MCF-7 cells experienced a structural integrity loss with disruption of length and development of isolated EC islands (Fig 1). The ECs appeared viable by trypan blue exclusion (data not shown). Because of the breakdown of architectural microvessel structure without loss of cellular integrity, we termed this phenomenon *microvessel dismantling.*

Because we have identified that HER2 signaling influences the induction of EC retraction in coculture with breast cancer cells expressing HER2, we compared MCF-7 cells with a MCF-7 derived cell line that overexpresses HER2 6-fold (HER cells) for the ability to induce microvessel dismantling in vitro. The MCF-7 treated cells showed a time-dependent decrease in cumulative length. At 3 hours, the treated microvessels retained only 72.3% ± 2.1% of control length ($P < .01$), which was reduced to 11.5% ± 2.6% of control by 12 hours ($P < .01$ vs 3 hours, Fig 2). HER cells induced a greater loss of microvessel integrity over time than the parental MCF-7 cells. At 3 hours, HER cell treated microvessel gels retained only 26% ± 0.8% of control length ($P < .01$ vs MCF-7), which was reduced to 4.6% ± 1.1% by 12 hours ($P < .01$ vs MCF-7, Fig 2).

To further evaluate HER2 signaling influence on microvessel dismantling, we pretreated MCF-7 cells with increasing doses of Herceptin for 24 hours before the microvessel dismantling assay. A dose-dependent blockade of MCF-7 induced microvessel dismantling was seen at 12 hours (Fig 3). Herceptin treatment (1.0 ng/mL) of MCF-7 cells reduced dismantling to 46.3% ± 7.1% of control ($P < .05$ vs untreated MCF-7). Increasing doses of Herceptin further reduced dismantling, reaching

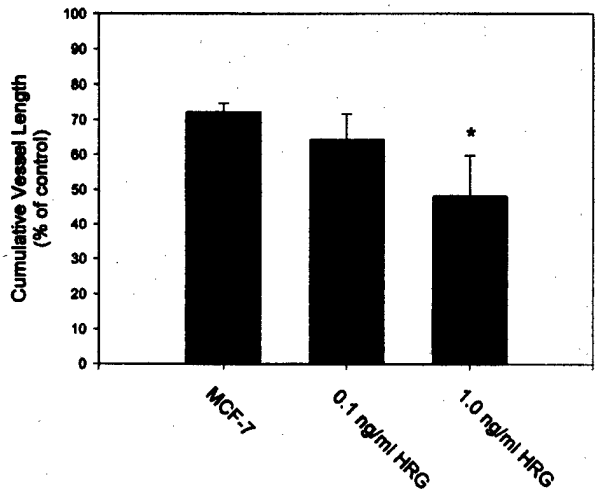

Fig 4. Stimulation of HER2 signaling augmented microvessel dismantling. The MCF-7 cells were pretreated with 0.1 or 1.0 ng/mL heregulin β1 for 24 hours before coculture with microvessel collagen I gels for 3 hours. Cumulative microvessel length was determined and compared with control. Treated cells were dose-dependently enhanced in inducing dismantling, reaching significance at 1.0 ng/mL (*$P < .01$ vs untreated MCF-7 cells; n = 3)

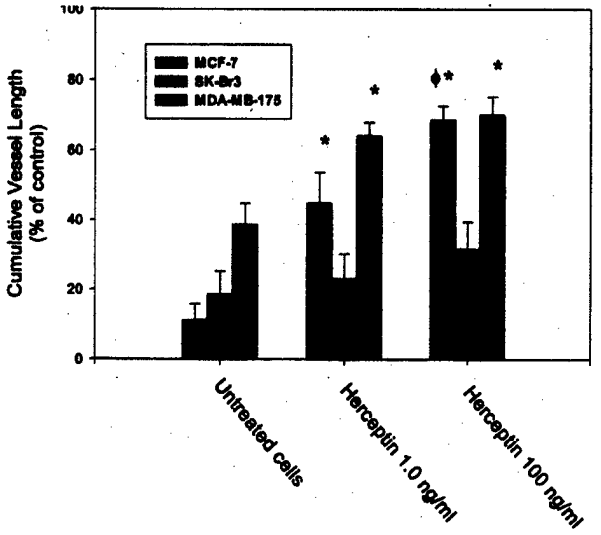

Fig 5. HER2 signaling induced microvessel dismantling in HER2 overexpressing cell lines. Untreated cells or Herceptin pretreated cells for 24 hours were placed in coculture with microvessel gels for 12 hours. Cumulative length of microvessels was determined and compared with control. All cell lines induced microvessel dismantling. HER2 signaling blockade with Herceptin inhibited induced dismantling in MCF-7 and MDA-MB-175 cells (*$P < .01$ vs untreated cells) but not SK-Br3 cells (P = not significant; n = 3).

68.6% ± 3.9% of control with 100 ng/mL ($P < 0.01$ vs untreated MCF-7).

Stimulation of HER2 signaling in MCF-7 cells using heregulin β1, resulted in accelerated microvessel dismantling (Fig 4). Cumulative length of intact microvessels at 3 hours was reduced to 48.6% ± 11.7% of control ($P < .01$) from 72.3% ± 2.1% after pretreatment with 1.0 ng/mL heregulin β1 for 24 hours before the microvessel-dismantling assay.

To determine whether other HER2 overexpressing breast cancer cell lines also could induce microvessel dismantling, SK-Br3 and MDA-MB-175 cells were cocultured for 12 hours with microvessel gels. Both cell lines induced microvessel dismantling with significant reduction in cumulative length of intact microvessels compared to untreated controls ($P < .01$, Fig 5). Pretreatment with 1.0 or 100 ng/mL of Herceptin for 24 hours reduced microvessel dismantling in MDA-MB-175 cells ($P < .01$ vs untreated cells). Pretreatment of SK-Br3 cells with Herceptin did not significantly reduce cumulative microvessel length at 12 hours of coculture.

The HER2 signaling has been shown to regulate Ang-2 production. Because of the profound effect of Ang-2 in inducing angioregression, we pretreated MCF-7 cells with sTie2 to sequester any Ang-2 protein released. By sequestering Ang-2, we attempted to implicate Ang-2 as a participant in the microvessel dismantling mechanism. The MCF-7 cells were pretreated for 24 hours with increasing doses of sTie2 before coculture in the dismantling assay. Sequestration of Ang-2 with sTie2 significantly though not dramatically reduced MCF-7 induced microvessel dismantling (Fig 6). At 200 ng/mL, cumulative length of microvessels cocultured with sTie2/Fc treated MCF-7 cells was 24.5% ± 5.5% of control ($P < .01$ vs untreated MCF-7 and MCF-7 treated with 2 ng/mL sTie2).

DISCUSSION

The HER2 overexpression induces a more aggressive phenotype in breast cancer cells. Early metastasis, earlier relapse, and a poorer overall prognosis accompany HER2 overexpression.[1-3] The finding that overexpression of a tyrosine kinase receptor induces a more aggressive phenotype empirically suggests that signaling leads to significant downstream gene modulation with gene product alteration. This gene product alteration likely must influence the local cancer environment, either directly or indirectly, to favor the more aggressive phenotype. In support of this hypothesis, Oh et al[17] identified several genes with expression levels in MCF-7 cells that were significantly altered by HER2 overexpression induced by transfection. Presumably, monoclonal antibody blockade of HER2 dimerization and signaling should

eliminate the selection of downstream gene expression, which favors the aggressive phenotype. Indeed, Herceptin treatment appears to lengthen remission episodes and delay return to an aggressive phenotype while rendering the cell static.[14,15]

Our prior studies have demonstrated that HER2 signaling regulates the induction of EC retraction with loss of cell-cell contact and the endothelial monolayer barrier.[16] The EC retraction creates a portal for transmigration across the endothelium and potential egress into a secondary tumor site. This process appears to be induced by secreted product or products regulated by HER2 signaling. In this present study, we have further characterized the vascular modulation induced by HER2 signaling. The HER2 overexpressing breast cancer cells may enhance metastatic capability by inducing an architectural disassembly of intact microvessels, creating a noncontiguous capillary network. The microvessels appeared to have disrupted EC contact with loss of continuity, a phenomenon we termed *dismantling*. This break in continuity suggests the possibility of tumor cell access into the vascular system and possible earlier disseminated metastasis.

We show that microvessel dismantling is at least partially regulated by HER2 signaling. Blockade of HER2 signaling resulted in a dose-dependent decrease in the ability to induce microvessel dismantling. Conversely, stimulation of HER2 signaling increased the cells' influence on microvessel integrity.

The process was not exclusive to MCF-7 cells. Both MDA-MB-175 cells and SK-Br3 cells also induced dismantling. The MDA-MB-175 cells were regulated similarly in this induction, shown by blockade of HER2 signaling with reduction in dismantling potential. The SK-Br3 cells were not influenced significantly by Herceptin blockade. It is possible that SK-Br3 cells process alternate or defective signaling or possibly influence different downstream gene transcription with different phenotypic expression. Xu et al[9] showed that the relative heterodimer expression could substantially change downstream phenotypic expression. By exogenously influencing relative levels of expression of HER3 to HER4 heterodimers with HER2, phenotypic expression shifted from a stimulatory pattern to an inhibitory pattern.

Because our prior studies have shown that HER2 signaling can regulate Ang-2 expression and further that Ang-2 appears to be involved in the mechanism of EC retraction induced by HER2 signaling, we postulated that Ang-2 may be the key factor involved in the induction of microvessel dismantling. Using sTie2/Fc to sequester Ang-2 released from MCF-7 cells, we showed a significant though not dramatic reduction in the loss of microvessel integrity. Unfortunately at this time, an adequate Ang-2 protein was not available to test directly. A crude preparation containing some intact Ang-2 protein was tested, but no significant induction of microvessel dismantling was identified (data not shown.) It appears that Ang-2 is likely a factor influencing the mechanism of dismantling, but is not the only or possibly even a major determinant of the process.

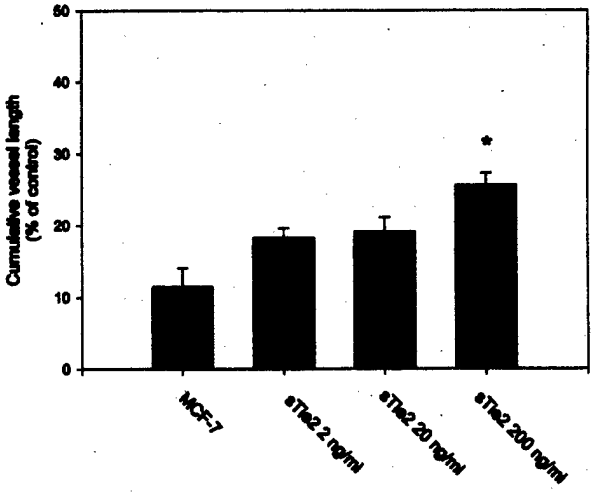

Fig 6. sTie2/Fc sequestration of MCF-7 produced Ang-2 inhibits microvessel dismantling. MCF-7 cells were treated with increasing doses of sTie2/Fc and placed in coculture with microvessel collagen I gels for 12 hours. Cumulative length of microvessels was determined and compared with control. sTie2/Fc dose-dependently inhibited induced dismantling, reaching significance at 200 ng/mL (*$P < .01$ vs control, 2, and 20 ng/mL; n = 3).

Microvessel dismantling also may be involved in an angiogenic response induced by HER2 signaling regulation of Ang-2 and other EC mitogenic factors. The Ang-2 expression in tumors occurs at sites of angioregression and capillary breakdown.[23] It appears that in angiogenesis, Ang-2 may influence EC-EC dissociation and release of anchoring, allowing mitogenic stimulation for proliferation and migration. The observed microvessel dismantling in this study essentially may be part of tumor co-option of microvessels with release of ECs, angioregression, and ultimately tumor induced angiogenesis.[24] In this scenario, discontinuity and possible angioinvasion may be a mere byproduct of tumor and environmental factors inducing an angiogenic response. The Ang-1 knockout mice, providing unopposed Ang-2 influence on Tie2 receptors, were noted to have discontinuous vascu-

lar networks with islands of isolated EC, offering some support for a role for Ang-2 in the dismantling mechanism.[19] Other factors such as urokinase plasminogen activator, a proteolytic enzyme, may influence both angioinvasion and tumor neovascularization in breast cancer by vessel wall degradation, providing a possible portal for tumor dissemination or release of EC for angiogenesis.[25]

In conclusion, we have demonstrated a phenotypic expression of HER2 signaling in MCF-7 breast cancer cells, induction of microvessel dismantling, which is likely stimulated by downstream expression of gene products favoring a metastatic profile. This process appears to be common to other HER2 overexpressing cell lines. The mechanism appears to involve cellular production of Ang-2, but clearly other factors that influence vascular structures are active in the mechanism. This study suggests that HER2 signaling induced metastatic pathways likely involve opportunistic expression of angioactive factors, which may provide a portal for angioinvasion.

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MECHANISM OF HER-2 SIGNALING INDUCED ENDOTHELIAL CELL RETRACTION

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Her2 signaling imparts a metastatic advantage in breast cancer. Her2 signaling in cancer cells induces coculutred endothelial cell (EC) retraction through a paracrine mechanism. Angiopoietin-2 (Ang-2), a factor that induces EC destablization, is upregulated by Her2 signaling in these cells. Because vascular endothelial (VE) cadherin complexes of the adherens junctions regulate EC-EC adherence, we postulated that EC retraction involves dissociation of the adherens junction proteins induced by tumor produced Ang-2.

Human iliac vein EC (HIVEC) monolayers were cocultured with estrogen-independent MCF-7 cells or a stable transfectant overexpressing Her2 6-fold (HER cells). Ang-2 protein was also directly tested against the monolayers. MCF-7 cells were pretreated with soluble Tie2 receptor fusion protein (sTie2/Fc) to sequester Ang-2 and test its role. Additionally, Her2 signaling was manipulated, using Herceptin to block or heregulin beta1 to stimulate signaling, to test whether catenin dissociation from VE cadherin was attributable to Her2 signaling. After 3 to 24 hrs, the monolayers were immunoprecipitated with anti-VE cadherin antibody. Western blot analysis was performed with specific antibodies against the catenins. Relative quantities of these proteins were determined and presented as % of untreated control monolayers.

A time dependent decrease in immunopecipitated catenins was seen in coculture with both cells ($P < 0.05$ vs control and between cells). Dissociation was accelerated by heregulin ($P < 0.05$, 100 ng/ml) treatment, or reduced by blockade with Herceptin ($P < 0.001$, 100 ng/ml). Ang-2 treatment induced extensive EC retraction ($P < 0.01$, 200 ng/ml), and dissociation of catenin. Pretreatment with sTie2/Fc reduced EC retraction ($P < 0.05$, 200 ng/ml), and blocked stimulated catenin dissociation ($P < 0.05$, 500 ng/ml).

These data indicate that the mechanism of Her2 signaling-induced EC retraction involves dissociation of the VE cadherin complex proteins, and likely involves Ang-2. EC retraction allows a portal for tumor transendothelial migration, a key step in metastasis. Blockade of Ang-2 binding to EC, and stabilization of the adherens junction protein complex may restrict the metastatic progression of Her2 expressing breast cancer cells.

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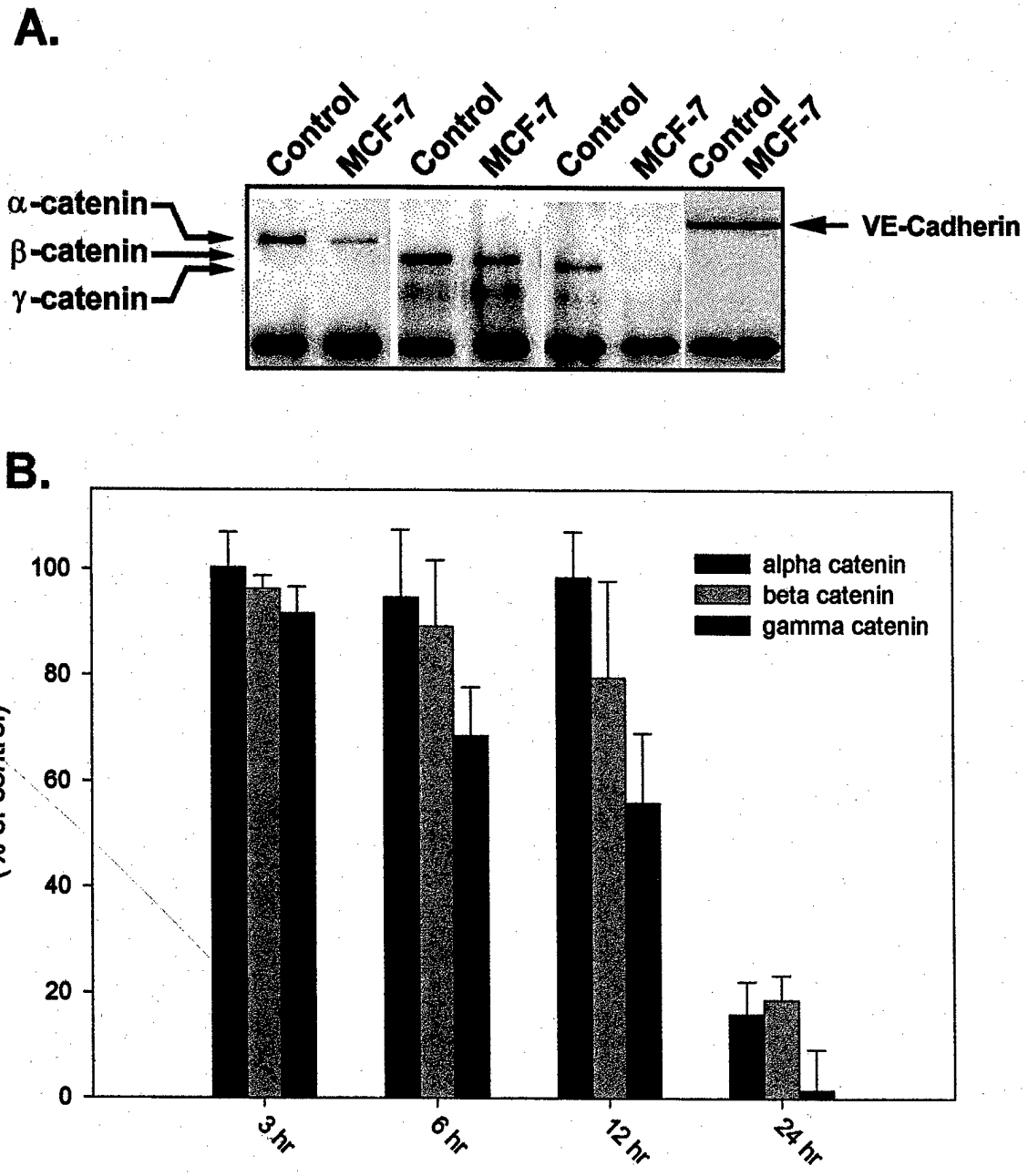

A.
Control
MCF-7
Control
MCF-7
Control
MCF-7
Control
MCF-7
α-catenin
β-catenin
γ-catenin
VE-Cadherin
B.
Densitometric intensity
(% of control)
100
80
60
40
20
0
alpha catenin
beta catenin
gamma catenin
3 hr
6 hr
12 hr
24 hr

Figure 1

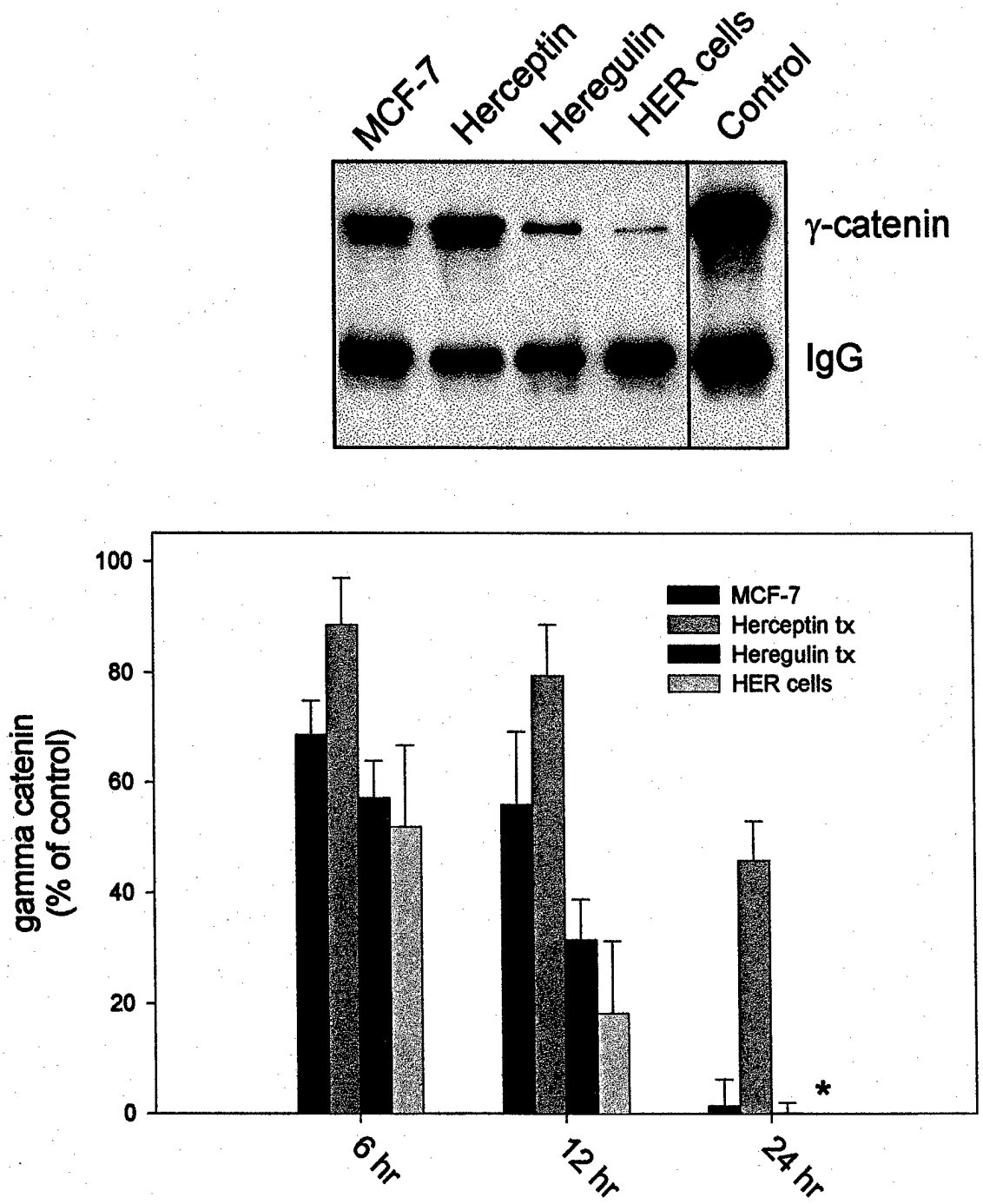
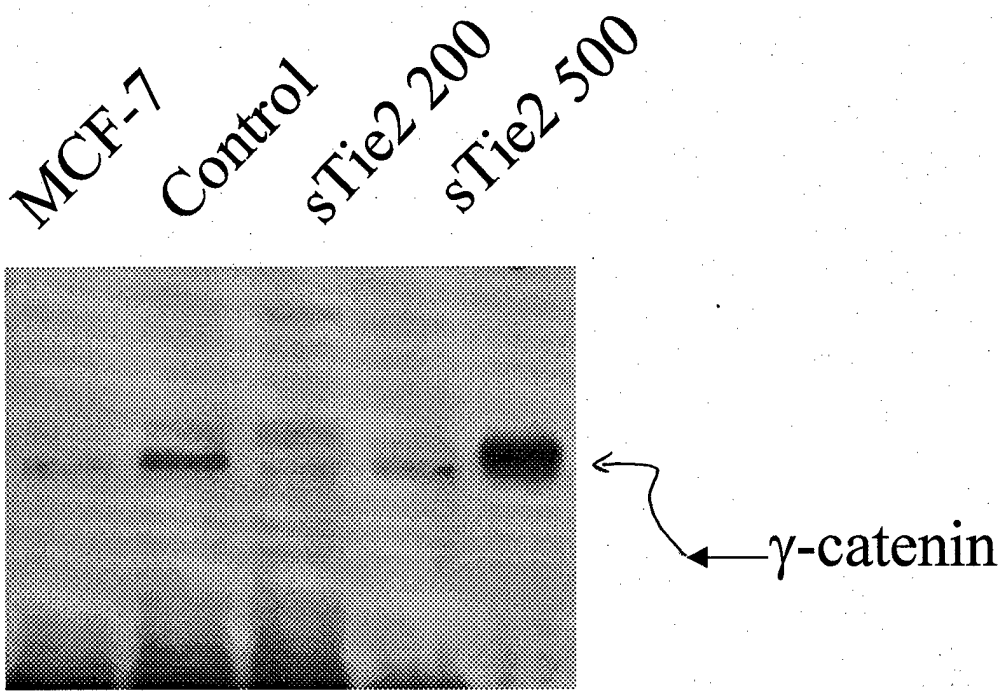

MCF-7
Herceptin
Heregulin
HER cells
Control
γ-catenin
IgG
gamma catenin (% of control)
100
80
60
40
20
0
MCF-7
Herceptin tx
Heregulin tx
HER cells
*
6 hr
12 hr
24 hr

Figure 2


MCF-7
Control
sTie2 200
sTie2 500
γ-catenin

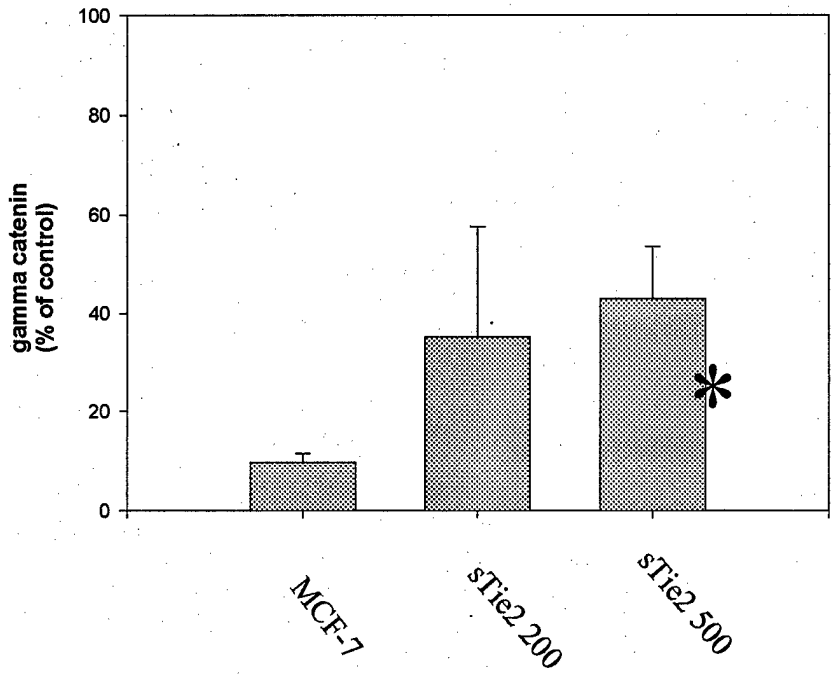

gamma catenin
(% of control)
100
80
60
40
20
0
*
MCF-7
sTie2 200
sTie2 500

Figure 3

Breast Tumor 002 RT-PCR

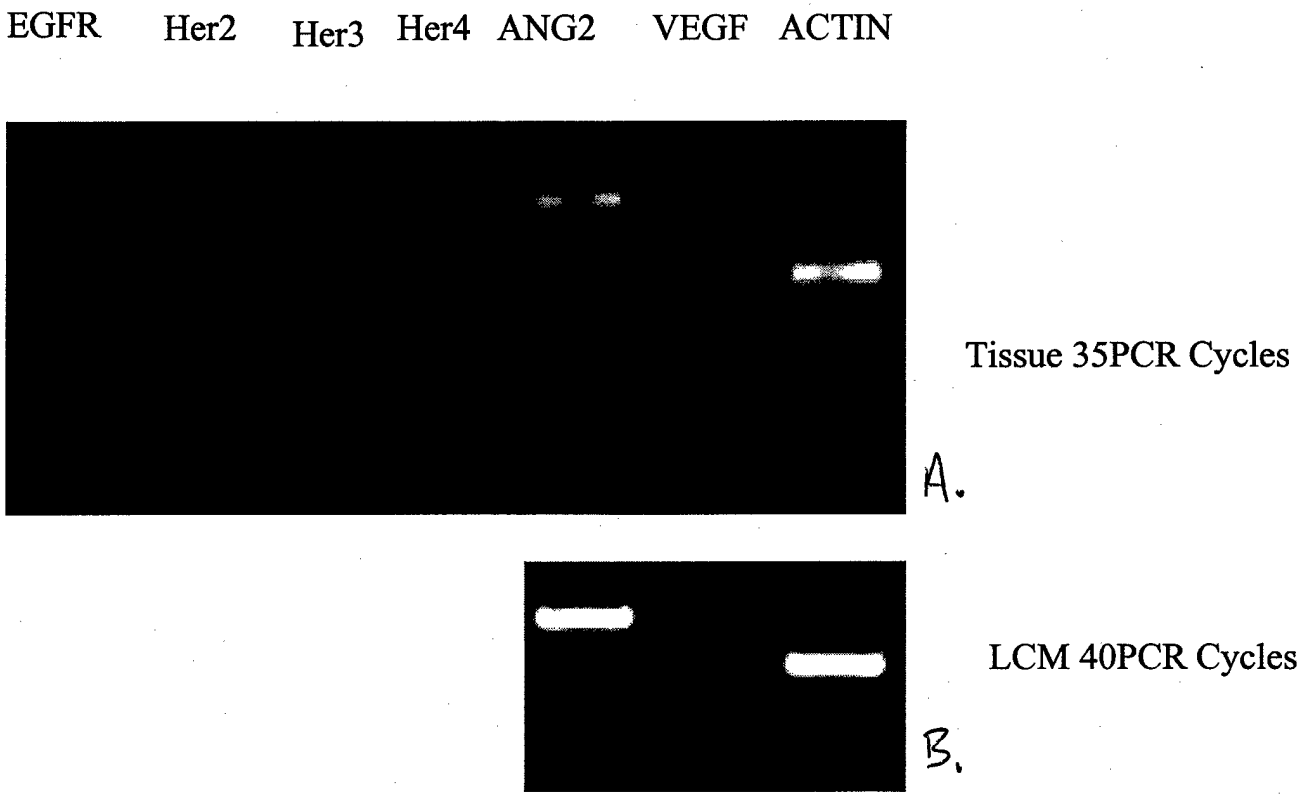


Figure 4

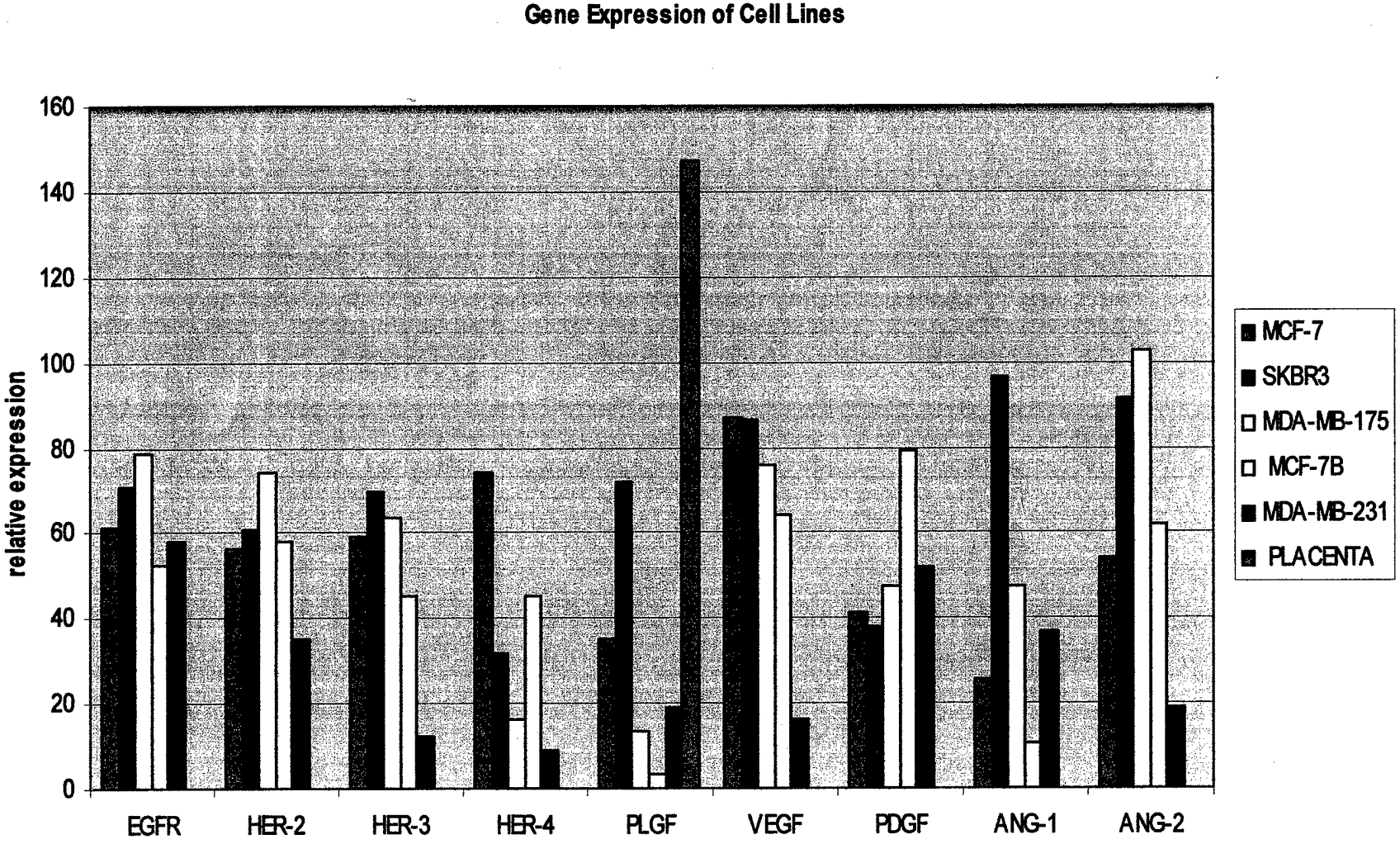

Gene Expression of Cell Lines
relative expression
160
140
120
100
80
60
40
20
0
EGFR
HER-2
HER-3
HER-4
PLGF
VEGF
PDGF
ANG-1
ANG-2
MCF-7
SKBR3
MDA-MB-175
MCF-7B
MDA-MB-231
PLACENTA

Figure 5

Human Angiogenesis GEArray of MCF-7 Cell Line

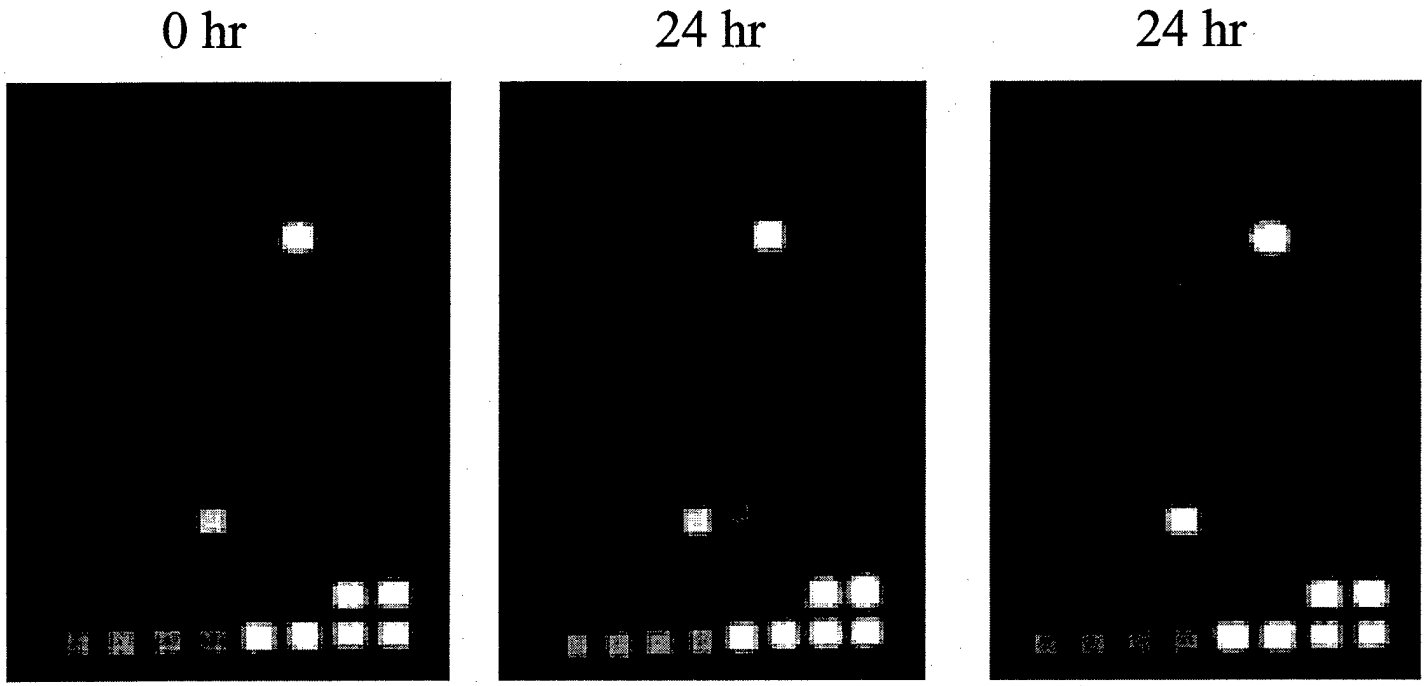


Figure 6

Human Angiogenesis GEArray of MCF-7 Cell Line HCT Treatment

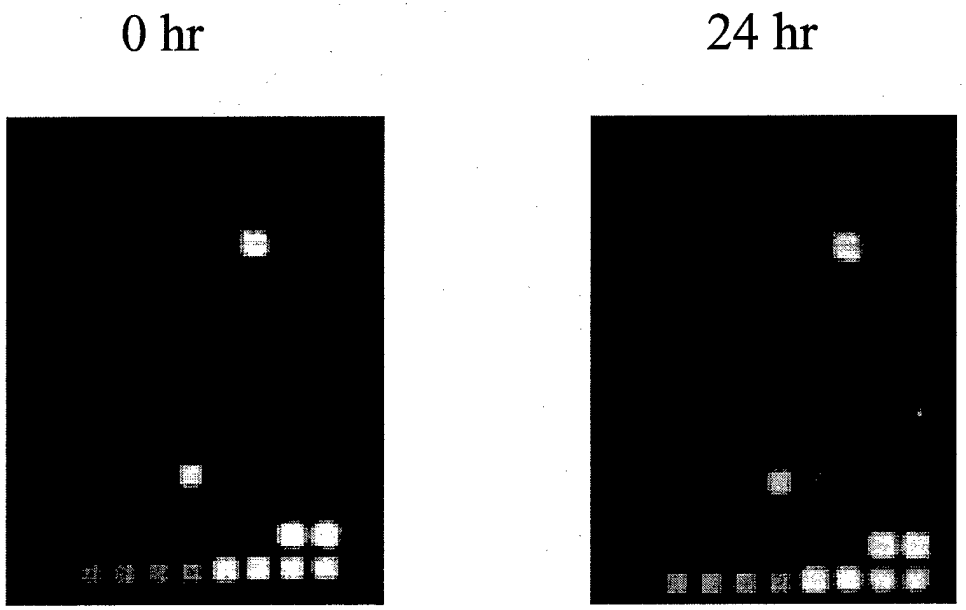


Figure 7